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THE RELATIONS OF BURSARIA TO FOOD

I. SELECTION IN FEEDING AND IN EXTRUSION

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EIGHT FIGURES

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INTRODUCTION

While studying the phenomena of regeneration and structural regulation in *Bursaria*, it became desirable to know something of the relations of this organism to its food and of the processes which the solid food material undergoes in its passage into and through the cell and in the elimination of residues. This paper aims to present the first part of these observations and to make a general survey of the relations of this organism to food, leading up to a more detailed study of these and certain other problems of the cell as found in this unicellular animal.

Bursaria was found to be much more favorable for the investigation of these phenomena than the smaller infusoria such as *Paramecium*. No study has heretofore been made of the relations of *Bursaria* to food, so that the facts herein presented are new. The present investigation attempts not only to determine qualitatively whether the relations to food are similar to those more or less known for *Paramecium*, *Stentor*, *Vorticella* and other infusoria, but more particularly to work out and express these relations in a more quantitative way than has been done heretofore. It was found that certain kinds of experimental tests such as those on the rate of digestion, could be made upon this form, which it would have been difficult or impossible to carry out on smaller unicellular organisms. Its very large size offers a singular opportunity for easy manipulation in many kinds of work. When in a clear medium it is readily visible to the naked eye at a distance of six or eight feet and individuals may be transferred singly with a pipette without the aid of any magnifying instruments.

Bursaria occurs not infrequently in cultures brought from ponds in the vicinity of Baltimore, though it is less common than many other Protozoa. It can readily be cultivated in large culture dishes in the laboratory. In this way I have had abundant material at my disposal for many months. The method of cultivation has been simply the inoculation of an infusion of timothy hay in tap water from the wild culture; by several inoculations at different times one usually succeeds in obtaining large numbers. Since the food of this organism is not bacteria,

so far as I have yet observed, but a variety of other ciliates, flagellates and rhizopods, it is difficult to find a culture medium which can be readily manipulated, and hence pure line cultures can not be obtained so readily as of a form like *Paramecium*. The problem of pure line cultivation of this organism will not be dealt with in this paper. The material for use in the following experiments has all been obtained from mixed or 'wild' cultures, though the reinoculations from the single parent culture brought into the laboratory seven months ago resulted in a small number of pure lines living side by side in the cultures.

It is, in fact, preferable in some ways to use material from such wild cultures for the kind of experiments to be considered in this paper.

Even without the aid of pure cultures or the application of statistical methods to wild cultures it soon became apparent that there are actually at least two very distinct races of *Bursaria* which differ in several diverse characters, physiological as well as morphological.¹ One form, which under certain food conditions has a tail, has been used exclusively in these experiments, since the other form, collected at the same time and lacking a tail, died out early in the experiments.

I have observed the following organisms to be eaten and digested by *Bursaria*: *Chilomonas*, *Colpidium colpoda*, *Vorticella* and some of its relatives, *Oxytricha*, *Stylonychia*, *Arcella*, *Stentor*, *Paramecium*, *Stephanodiscus*, and some kinds of rotifers. Only once have I observed bacteria to be eaten, and that time in the form of zoogloea. It is, however, certain that bacteria form only a small part, if any, of the usual diet of this organism. The smaller ciliates, flagellates and rhizopods are the favorite article of food. The larger organisms, such as *Stentor*, are seldom successfully captured. *Paramecium* is quite commonly eaten, though *Bursaria* does not seem to thrive well on this food. Occasionally rotifers are eaten and it was observed on several occasions that these may remain alive within the vacuole

¹ I have been unable to find reference in the literature to more than one form of *Bursaria*. A consideration of the problems connected with the existence of diverse 'races' of *Bursaria* will be left for a later time.

for as long as five hours before they are killed. It is, however, plainly evident when one follows the development of wild cultures from day to day that some forms are eaten in greater numbers than others and if the smaller forms, such as Colpidium, Vorticella and Arcella, are present in abundance along with such forms as Paramecium, Stentor and Stylonychia, the former kinds serve exclusively as food for Bursaria while the latter are rarely eaten. When the cycle of development of the culture comes to the stage where, for example, Paramecium is in superabundance, then the body of Bursaria may be more or less filled with Paramecia. In contrast to the above mentioned forms, Spirostomum ambiguum was always rejected. It was often seen to be taken into the oral pouch but invariably was thrown out again, while Paramecia present in the same culture were readily eaten by the same Bursaria individuals at the time of the observations. This is the only case where Bursaria was seen definitely to discriminate between two different forms of Protozoa.

By simple methods of observation like the above, it would be impossible to determine just what the principle and the factors are that determine whether Bursaria will feed on only one or several or all of these forms if they be present in all the cultures simultaneously, which of course they often are. It is with the object of elucidating these and certain related questions that the following comparatively simple experiments have been performed, by limiting and determining to a high degree the conditions under which this organism will react to food.

ACTION OF THE STRUCTURAL MECHANISM FOR FEEDING AND THE SELECTION OF FOODS

An account of the food relations of Bursaria requires us to examine in some detail the objective processes involved in feeding; these are very striking. The highly developed oral apparatus with its large cilia, when in operation during the feeding process, may easily be observed. When the organisms are fed on such substances as yolk or starch they usually sooner or later become quiet for a time, and settle to the bottom of the dish or stick to the surface film of the water, then they may be

observed under a high power of a binocular. Granular substances of different chemical or physical properties may be placed in the medium and the path of each individual particle may be easily observed. Mixtures of these substances may also be made and the paths of the different kinds of particles may be determined.

The different paths of particles which come into varying relations with the organism are shown by the arrows in the outline drawings of figure 1. The paths of the arrows are correct representations of the paths taken by the different kinds of particles. In general the paths taken by particles may be distinguished according to the following outline:

1. Paths of rejection

a. Path of total rejection, arrows A

b. Path of rejection of larger particles taken into the oral apparatus, arrows B

c. Paths of rejection of smaller particles taken into the oral apparatus, arrows C_1 and C_2 .

These paths may also be slightly modified by a combination of the avoiding reaction with the different rejection reactions.

2. Path of acceptance of large and small particles (large arrows D)

Path A is taken by those particles which under conditions hereafter to be considered (p. 29) never enter the oral apparatus and are only drawn towards the body by the current; for example, very toxic particles of yolk. Path B is always taken by those particles which are too large to pass out by way of path C_1 and C_2 and must be passed back to the exterior by the same way as they were taken in, in order for the organism to get rid of them at all. This may be illustrated by the larger properly treated grains of hard boiled yolk. The path represented by the arrows C_1 and C_2 has considerable range of variation in part of its course. It may be illustrated by cornstarch grains; these are of convenient size. The variations in the course of these particles may be divided roughly into two main divisions; some follow the dotted arrows C_2 and never directly retrace any

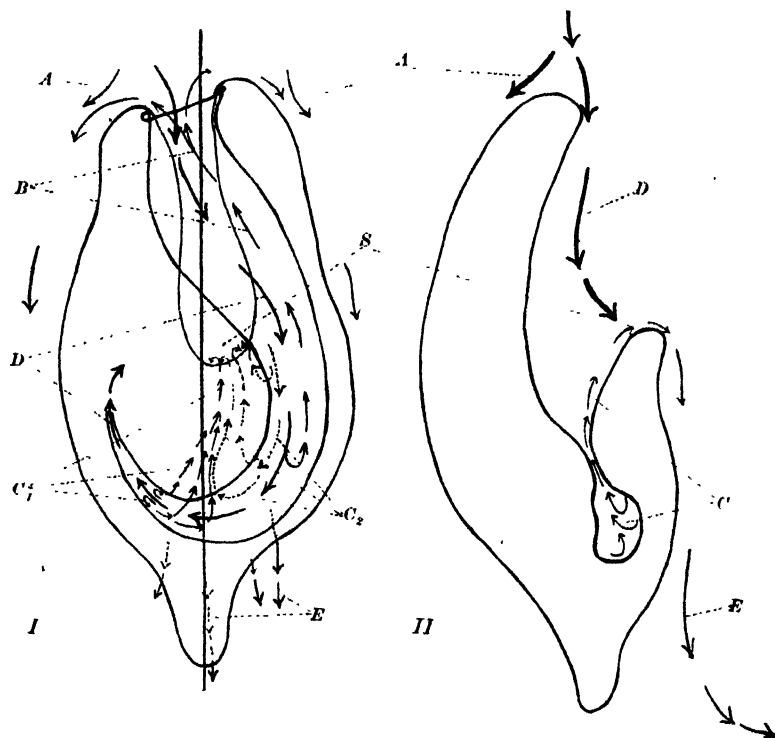


Fig. 1 *I*, Outline drawing from dorsal side of *Bursaria*, to show position of the oral pouch in the body, and the paths of variously rejected and accepted particles. *A*, path of total rejection; *B*, path of rejection of large particles which are too large to pass out by way of the oral sinus, *S*. *C*₁ and *C*₂, paths of rejection of small particles; these pass out by way of the oral sinus, *S*; *D*, path of acceptance. *II*, Outline drawing of sagittal section, in the plane through the body represented by the straight line through *I*. *A*, path of total rejection; *D*, path of entrance of all particles taken into the oral pouch, same as first part of path *D*, *I*. *C*, path of rejection of small particles, same as *C*₁ and *C*₂ of *I*. *E*, *I* the same as *E* *II*, direction of rejected particles, *C*, *I*, and *C*₁, *C*₂, *II*.

part of their former path; some pass up into the proximal end of the oral pouch but are rejected and returned to the outside by way of the continuous arrows *C*₁. All the paths of rejection under *C*₁ and *C*₂ converge and lead to the exterior by way of the oral sinus, figure 1, *I* and *II* *S*.; they then pass backward under the posterior ventral side, as shown by arrows *E*. There is but one path of acceptance for both large and small particles.

Figure 1, *II*, is a sagittal section through the body in the plane indicated by the straight line through figure 1, *I*; it shows the path of total rejection, arrows A, the path of entrance (by heavy arrows D) and the path of rejection of smaller particles (by arrows C').

At the point of entrance into the endoplasm the transport of the accepted particles is brought about not alone by the cilia but also if not exclusively, in the case of larger particles, by a peristaltic wave in the wall of the oral cavity behind the particle pushing it into the body.

FORMATION OF THE VACUOLE AND THE ELIMINATION OF RESIDUES

The vacuoles when formed always contain some liquid, though at times the amount may be very small. The size and shape of the vacuoles varies greatly and depends upon the kind of food eaten, and upon many other conditions, as will be shown. Often the food forms large irregular masses, which in the case of fresh yolk may so completely fill the body that after a half-hour or more of feeding the dorsal side of the body cortex is burst open and the food mass is extruded. The opening then closes and the organism again assumes its usual shape. The rate of formation of the vacuoles is intimately bound up with the same complex conditions which determine their size and shape. The circulation of the vacuoles in Bursaria is not reducible to any definite order, such as has been shown to exist more or less definitely in *Paramecium*, by Metalnikow ('12) and others, and in *Carchesium* by Greenwood ('94). The vacuoles often become lodged in one place and there digestion is completed. This may often be seen in cases where fat-extracted yolk particles become lodged in the tail. During digestion and resorption the large vacuoles usually become smaller and any residual contents are finally extruded. The residues are always extruded from a small area on the mid-dorsal side of the body of the organism. This may readily be demonstrated by feeding the animals chinese ink. The changes which take place in the food vacuole from its formation to its disappearance will be considered in detail in a later paper.

MEASUREMENT OF THE AMOUNT OF FOOD EATEN AND METHOD
OF EXPERIMENTATION

In order to express quantitatively the relations of *Bursaria* to food, it is necessary to obtain a reliable method for measuring the amount of food taken in a given length of time, under given conditions. The unit of volume employed was that of one grain of fresh hard-boiled yolk of hen's egg. The eggs were boiled fifteen minutes. These grains are readily eaten by *Bursaria* and may be obtained of an approximately uniform size. It is necessary to deal with suspensions of such grains, having a uniform concentration (that is, containing the same number of grains to a given volume). For this purpose stock suspensions were made up on successive days from yolk of the same egg: these were made uniform by making them up in vials of the same size and comparing each with a standard concentration kept in a vial of the same size. Various known grades of concentration were then made up by adding a known volume of the stock suspension to 5 cc. of water in a slender dish of 8 cc. capacity. This procedure was found to be sufficiently accurate to avoid the introduction of any observable variation in the amount of yolk eaten in a measured period of time (page 20.²). Uniformity in the size of the yolk grains was obtained by repeatedly washing the fresh hard boiled yolk crystals in distilled or tap water and decanting, until the suspension when left to settle leaves a clear supernatant liquid. The smaller grains remain in suspension a little longer than the larger ones and thus may be removed by decantation. Uniformity in size is still further obtained by drawing off the grains from the same level in the clear suspension with a pipette. Some eggs have yolk crystals of more uniform size than others, so that only the eggs best in this respect have been used.

² In most of the experiments it was necessary to make up only a single stock suspension, since the animals were fed only once and all the feeding was carried out at the same time. In the case of experiments which required the feeding of yolk on more than one day, however, this standard concentration had likewise to be made up anew each day by comparison with that of the day before.

The uniformity in size of the yolk grains is of course of paramount importance in many of the experiments and for some of the conclusions which will be drawn from them. In order that the degree of uniformity might be tested and indicated quantitatively, a large number of measurements of grains of the prepared yolk suspension were made at different times by means of a stage micrometer. The following shows a typical result of one set of these measurements: 105 grains were measured and the numbers divided at random into three sets of 35 each and the average of the diameters taken. These gave respectively 0.0890, 0.0906, and 0.0837 mm. The range of variation of the diameter was from 0.060 to 0.130 mm. The distribution of the variations are shown by the following figures:

Diameter of grain mm....	0.06	0.07	0.08	0.09	0.10	0.11	0.12	0.13
Frequency.....	12	19	16	14	20	13	6	5

By thus being able to obtain a very constant average diameter of a comparatively small number (30 to 50 grains) the errors introduced by the individual variation in size, which in the above example is about as $\frac{1}{2}$ is to 1, is largely eliminated. In order to remove the objection to experimental results based on the volume of granules of varying size, a large number of individuals (20 to 100, depending upon the purpose of the experiment) were used in each experiment and the number of grains eaten was counted; furthermore the experiments were always repeated whenever there could be any doubt as to the validity or significance of the results. Hence, as will be shown later, the lack of strict individual uniformity of the unit volume is corrected (a) by the fact that the average size of the yolk grain is practically constant, (b) by using a large number of individuals in each experiment, and (c) by repeating the experiment.

Thus having the form, weight and volume of the units of food eaten made practically constant, we may vary one of their properties—as for example, their chemical nature—by letting them adsorb different kinds of toxic and non-toxic substances which are diffusible or non-diffusible in the native medium, tap or distilled water. We may therefore test the responses to variations

in this one property—namely, the chemical nature of the grain—and its effects.

An approximately constant medium was provided by using tap water. This precaution is important, for, as will be shown, the nature of the medium often affects or determines the kind of results which are obtained. Distilled water was also used but it was found that this extra precaution was not necessary in most of the experiments, and since distilled water is toxic if the organisms are left in it too long or the change is too rapid, it could not have been used in many of the experiments, even if it had been otherwise desirable to do so.

The organisms were starved in 400 cc. of tap water for eighteen to twenty-four hours previous to each experiment. At the end of this time they were free from food and residues. Thus an optically clear, active and perfectly normal cell was obtained with which to begin work in all the experiments where uniformity in this respect was desired. All the factors with which we are dealing except the 'physiological states' of the organisms themselves are known and uniform to within narrow limits, while the one of which we wish to test the effects can be controlled and varied.

INTERNAL RELATIONS AFFECTING THE FEEDING PROCESS

1. The relation of the physiological state of the organism to the feeding process

By the words 'physiological state' is here meant the condition as a whole, of the equilibria in the physical and chemical reaction system, the cell, at a certain time in the duration of its existence.³

This condition or state is to be thought of as being limited to the space which the organism occupies, or is, in other words, internal. However, it is obviously absurd for anyone to attempt

³ This definition is justified because in so far as the facts are at present known, this is the only kind of system with which we have to deal in the cell, and therefore in the present state of knowledge the only logical universal assumption for experimental purposes is to define 'physiological states' in terms of such known systems, until the universality of the assumption is disproved.

a definite and strict separation of the internal and external of any living organism, and especially is this true of the cell. Yet for purposes of presentation, this becomes highly convenient, and it is only for this purpose that the above rough distinction is made here. *When all external conditions are made the same in two experiments which nevertheless give different results, the differences must be attributed to different conditions within the organism,* and it is, as a rule, only in this way that different physiological states are at present practically perceptible.

Differences in physiological state in unicellular animals are made evident most readily in the relations to food, as may be seen from the work of Metalnikow ('12) on *Paramecium* and by Schaeffer ('10) on *Stentor*.

Bursaria affords most excellent material for the elucidation of the relation of these dynamic states to the feeding process and of the fact that this relation changes while the external conditions remain constant. These facts are brought out in the following experiments by using both single individuals, and large numbers of individuals collectively, at the same time, and analyzing the results.

The total quantity eaten and the rate of feeding. Table 1 gives the results of a typical experiment designed to show the difference in the total quantity of food eaten and also the difference in the rate of feeding of Bursaria from different cultures.

Material from two different cultures, *A* and *B*, was starved twenty-four hours in each of two dishes containing 400 cc. of tap water. 1 cc. of a fresh hard boiled yolk suspension was placed in each of 16 stender dishes of 8 cc. capacity; 5 cc. of tap water was then added to each. Thirty individuals from culture *A* were placed in each of 8 of the dishes. Alternately with these 8 sets from culture *A* were placed 8 sets of thirty individuals each from culture *B* in the other 8 dishes. At the end of the time intervals noted in the table, in each case, the contents (6.5 cc.) of one dish each of *A* and of *B* were transferred into a stender dish with 500 cc. of tap water. This stops the feeding. The individuals were then immediately picked out of these large dishes, placed in 8 cc. dishes and killed in Meves' fluid. The

counts of the number of grains contained in each individual were taken at the end of the experiment.

Table 1 shows (1) that *Bursariae* living in the two different cultures differ in the total amount of food eaten in the same length of time. In other cases, of course, individuals from diverse cultures will give identical results so far as feeding is concerned, while two or more different cultures may also differ to a greater extent than the above table shows. Moreover, the amount of food eaten by a given culture may vary at different times. The greater the length of time of feeding (within certain limits) the greater the total amount of food eaten. Not only does the total amount of food taken differ in the two cultures, but what is equally important, (2) the rate of feeding varies with organisms from different cultures. This was observed in numerous other experiments. Under some conditions the animals fill their bodies quickly, while at other times this takes place slowly; or only a small number of grains may be eaten.

The facts are shown most clearly by the curves *A* and *B*, figure 2, representing the number of grains of yolk (ordinates) eaten by the thirty individuals in successive periods of one-half minute (abscissae) throughout the time of the feeding process. Curve *A* is plotted from the results of culture *A* and curve *B* from those of culture *B*, in table 1. The immediate rapid rise of curve *A* shows that the rate of feeding of culture *A* during the first six successive periods of one-half minute each was about from five to twenty times as great as in any of the subsequent fifty-seven minute intervals. A similar high initial rate is also shown by curve *B* (culture *B*), but here the rise to the maximum was not so steep and the rate during the first six half-minute periods was only about from four to ten times the rate during the subsequent fifty-seven half-minute intervals.

In order to show more clearly that the results apply to the individuals taken separately as well as to the averages for all (i. e., to the cultures as a whole) the data may be arranged as in table 2. As this table shows, at the end of sixty minutes all but an insignificant number of animals from each culture had eaten yolk grains: hence, the difference in the amount and

TABLE 1

Showing the results of a comparison of the rate of feeding and the total number of grains of yolk eaten by eight sets of thirty individuals each from two different cultures, A and B

TIME FED IN MIN- UTES	CUL- TURE	NUMBER OF GRAINS OF FRESH YOLK EATEN BY EACH CELL																							TOTAL NUMBER OF GRAINS EATEN	AVERAGE NUM- BER OF GRAINS EATEN PER INDIVIDUAL	AVERAGE NUMBER OF GRAINS EATEN IN SUCCESSIVE 1/2 HOURS	AVERAGE RATE OF FEEDING						
1	A	0	0	1	0	1	1	2	3	0	1	0	4	0	0	2	2	1	2	1	0	1	0	2	3	0	32	1.06	32.0					
	B	0	0	1	0	0	0	1	0	0	0	1	2	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0.33	10.0					
1	A	1	2	1	0	1	0	4	0	6	1	1	4	6	0	2	1	0	1	6	2	4	4	8	6	3	0	4	2	0	70	2.33	38.0	
	B	0	0	0	1	4	0	1	0	1	2	0	2	0	0	3	0	3	0	0	1	1	0	0	2	0	4	0	25	0.83	15.0			
3	A	10	9	16	20	9	8	3	5	8	6	10	11	2	0	1	5	7	4	0	6	3	1	0	12	0	13	4	7	6	12	198	6.60	32.0
	B	0	1	3	1	0	1	0	1	0	3	0	0	2	2	4	0	7	4	0	2	1	3	6	4	3	2	0	4	38	1.93	7.0		
5	A	0	0	10	11	10	0	0	9	12	11	0	15	9	12	5	6	4	10	11	7	0	15	10	8	6	7	8	12	11	8	227	7.56	7.25
	B	3	3	4	6	1	4	3	2	3	4	0	2	0	1	0	3	0	2	1	0	3	2	4	0	2	6	2	5	1	4	71	2.37	3.25
10	A	9	24	7	10	8	4	9	5	16	6	11	12	3	10	8	4	8	3	0	3	16	3	14	16	4	7	0	12	9	241	8.03	1.4	
	B	10	7	7	4	3	0	4	0	2	3	2	9	2	3	3	2	0	0	5	1	3	1	2	3	1	1	6	3	0	91	3.03	2.0	
20	A	14	10	10	15	3	7	10	13	7	4	11	7	0	7	3	2	10	9	15	12	18	12	15	5	14	19	8	9	2	292	9.73	2.55	
	B	1	1	6	8	1	0	4	3	6	3	0	1	6	1	1	9	9	3	8	0	8	3	4	9	0	0	2	114	3.80	1.15			
30	A	15	12	16	24	15	0	7	14	5	7	4	16	7	24	3	16	13	15	11	16	0	10	0	9	20	2	4	14	215	10.53	1.2		
	B	6	3	6	0	7	6	2	4	5	2	0	5	4	5	1	0	8	4	6	5	9	4	6	5	9	4	0	6	5	4	117	3.90	0.15
60	A	16	0	115	8	19	0	8	20	5	23	6	12	13	29	9	11	14	6	13	22	13	24	15	16	11	0	19	381	12.70	1.08			
	B	6	8	8	14	10	3	8	16	4	16	0	3	0	1	5	4	8	3	8	9	0	6	1	0	12	0	5	0	180	6.00	1.05		

rate of food taken by the two cultures was not due to some sporadic difference caused, for example, by a very high rate of feeding by a few individuals and no food eaten by others, but rather to a uniform difference between the sets of individuals from the two cultures. Therefore the results are typical for the individual as well as for the culture as a whole. Moreover, if we calculated the averages of *A* and *B* on the basis of those individuals alone which had one or more grains, the average of *A* would still be greatly in excess of that of *B*.

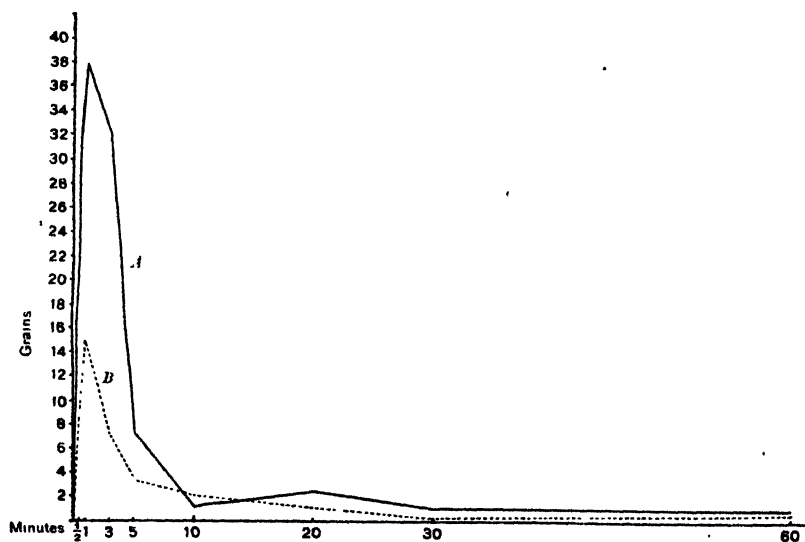


Fig. 2 Showing the rates of feeding by the two cultures *A* and *B*, curves *A* and *B*, respectively. Plotted from the results of table 1.

We may express the variation in the total quantity eaten by the standard deviation of each corresponding group of thirty individuals in *A* and *B*, as is done in the last column of table 2. The reciprocal of the standard deviation (σ) is a measure of the degree of uniformity among the individuals. It will be noted that there is an increase in the range of variation and the standard deviation with increase in the length of time of feeding; this means that the difference in physiological state among individuals of the same culture finds a fuller and more definite ex-

pression in the results of the experiment as the length of time of feeding is increased. The final total number of grains eaten when the time is long is then a more accurate index of the relation of the physiological state to the feeding process than if the time of feeding is short. The greater this difference in the total quantity of food eaten the greater is the difference in the physiological state of the different individuals. We, therefore, have in the amount of food eaten, if the length of time of feeding is long enough, a fairly good relative measure of the physiological state of the single individual and the differences in the physiological state between different individuals as regards their relation to food at that particular time.

2. Changes in the physiological state as shown by using the feeding process as an index

If change in the dynamic conditions of the cell, as regards the food relation, does occur, this should be observable by a change in the feeding process, and such is indeed the fact. This is shown in table 3. Material from cultures *C* and *D* was starved in tap water for twenty-four hours. Five active individuals were then picked out from each and tested individually. They were fed twenty minutes and each one was observed continuously during the experiment. The number of grains eaten and rejected and the time as called off by the observer were noted.⁴ In this way the time record of the relation of acceptance and rejection of food was obtained. The yolk concentration, temperature, and so forth were the same in all the tests.

As the table shows, yolk grains were at first rapidly eaten. At the end of the first few one-half minute intervals the action of the cilia was frequently reversed, thus rejecting the food after it had been taken into the oral apparatus. There was, therefore, a definite change from eating to the rejection of food by the feeding mechanism. This change was more rapid in general, in the individuals from culture *C* than in those from culture *D*.

⁴ I am indebted to Mr. K. S. Lashley for kindly aiding me in taking the records of this experiment.

TABLE 3

April 4: CULTURE C
Starved, tap, 24 hours

Tests on individuals

TIME MINUTES		1	1½	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	SUM								
I	Accepted...	5	1	3	3	3	6	4	5	3	3	6	7	11	7	1	4	3	1	7	2	2	5	6	7	3	2	6	1	4	118
	Rejected...	4	3	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	11	
II	Accepted...	3	4	7	3	3	2	6	4	4	1	2	1	4	3	2	3	1	3	2	4	5	2	2	4	5	2	2	4	5	74
	Rejected...	3	4	7	3	3	2	6	4	4	1	2	1	4	3	2	3	1	3	2	4	5	2	2	4	5	2	2	4	5	21
III	Accepted...	4	1	2	4	1	2	2	4	3	6	1	3	1	1	2	2	2	2	1	1	3	1	2	2	2	1	1	2	2	60
	Rejected...	3	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	11	
IV	Accepted...	5	6	1	3	3	3	2	2	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	57	
	Rejected...	5	6	1	3	3	3	2	2	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	15	
V	Accepted...	1	4	3	2	3	3	2	2	1	2	1	1	1	3	2	2	1	1	2	3	1	1	1	1	1	1	1	1	50	
	Rejected...	1	4	3	2	3	3	2	2	1	2	1	1	1	3	2	2	1	1	2	3	1	1	1	1	1	1	1	1	1	

April 5: CULTURE D
Starved 24 hours

I { Accepted	2	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	11
I { Rejected	2	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	32
II { Accepted	2	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	14
II { Rejected	3	4	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	47
III { Accepted	3	4	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	17
III { Rejected	3	4	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	43
IV { Accepted	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	8
IV { Rejected	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	8
V { Accepted	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	17
V { Rejected	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

These results from individuals are therefore strictly comparable and in accord with those obtained when a large number are tested at one time (table 1).

Now, in order to explain the cause of the change in reaction the suggestion might be offered that *Bursaria* shows a decrease in the rate of feeding because of the decrease in the amount of space in the body which food can occupy. This is undoubtedly true to some extent in those individuals which do not stop feeding until the cell becomes distorted by the comparatively immense mass of food. So far as the volume capacity of a normal individual of *Bursaria* is concerned, hundreds of observations have shown me beyond doubt that this may frequently be as much as *twenty-five to thirty grains*. Nevertheless, reversal of the cilia always takes place sooner or later. But the suggestion evidently does not apply to those individuals which show a change in the reaction when only a few grains have been eaten, for it seems impossible to understand how there could be a difference of as much as twenty grains of fresh yolk (table 2) in two normal individuals of equal size, when the cells are under exactly the same conditions, if this result were not due to a difference in the physiological state of the cells. Change in feeding was caused by the periodic reversal of the cilia and the reversal of the cilia in turn in some manner initiated or caused by a stimulus from the food already eaten, for it seems most natural to suppose that the stimulus originated from the change produced by the food mass in the interior of the cytoplasm. The most definite evidence that the change is due to stimulus from the eaten food is found in the radical change in the action of the cilia of the feeding mechanism.

If such fed individuals as those in table 3 are left in tap water free from food they may again eat yolk after digestion is partially or wholly completed, and again show a similar decrease in the rate of feeding, that is, a reversal of the oral cilia. The total quantity which will be eaten may be greater than that eaten at the previous feeding; but it usually is less, or often none at all.

The process of feeding in Bursaria shows it to be a functionally equilibrating system in its behavior towards food and the condition of its equilibrium at any particular time constitutes the physiological state which the cell is in, so far as its relation to food is concerned. The changes in the increase or decrease in the quantity of food eaten in successive meals and the increase or decrease in the rate of feeding might be discussed in the psychological terms 'hunger' and 'satiation;' but it is evident that the simpler terms quantity and rate express the facts of experiment, while any attempt at definitely determining whether the changes in quantity and rate are the same or different from 'hunger' and 'satiation' will obviously lead nowhere. Hence it seems better to use the terms, rate and quantity, which have a clear and quantitative meaning.

3. Other causes of individual variation

Bursaria at times closes up its oral apparatus. This may take place to such an extent that the opening is smaller than the food particles and then the latter can of course not be eaten. This condition can readily be observed under the binocular and it can always be determined beforehand whether closure has taken place to such an extent that the organisms can not feed. Other minor accidental individual variations are also present to some extent. These may be partly due to the difference in the size of the grains of yolk eaten. Sometimes when an individual is weak, owing to prolonged starving or for some other reason, two or three grains may become stuck in the oral pouch and this prevents feeding until the animal succeeds in throwing them out or by other means they become loosened. The material used was always examined beforehand to make sure that it was in a healthy condition so that these accidental conditions play no part in the final results of the experiments described.

Such a series of experiments as the foregoing do not show us specifically what these complex conditions are which have been cloaked in the phrase 'physiological states.' This however is

not the object of the above experiments: they are only here considered for the purpose of demonstrating the existence of these conditions, the fact of change within them and especially in this connection their rôle in the *external* phenomena of feeding and food selection in *Bursaria*, and how they may affect the results which will be given in the following pages.

EXTERNAL RELATIONS OF THE FEEDING PROCESS

1. *Effects of external factors on feeding*

a. Concentration of the food supply. The rate of feeding is within comparatively wide limits not dependant upon the concentration of the yolk suspension, provided it is not too low. This may be illustrated from one out of a series of experiments. The time of feeding was reduced to five minutes for the purpose of bringing out the effect of difference in the concentration more strongly. If the animals had been left in the suspensions twenty minutes (the usual time of feeding; cf. table 3) the difference would have been less marked, especially with material which shows a high rate of feeding.

*Experiment I.*⁵ Material from a healthy culture was starved twenty-four hours in tap water. All were perfectly normal and active. The experiment was carried out in 8 cc. stender dishes. The concentration in dish *B* was 8 times that in dish *A*. Twenty individuals were placed in each dish. The results from trial number 2 represent more nearly the ideal because these two suspensions were kept uniformly distributed during the five minutes feeding, and the individuals were picked out alternately by fives. Both trials, however, express equally well the proportional effect of concentration, namely, 1 to 2, as compared to the proportion of concentration, 1 to 8⁶ (table 4).

The concentrations used in the experiment are approximately represented by figure 3.

⁵ The experiments given in this paper are numbered in regular order for the convenience of the reader, and do not represent the actual order. Only a small number of the experiments actually carried out are given.

⁶ In all the experiments considered in this paper, where the concentration plays a part, the concentration was intermediate between those used in this experiment (fig. 3)

TABLE 4
Experiment I

TRIAL	DISH	NUMBER OF YOLA GRAINS IN EACH INDIVIDUAL																				TOTAL	AVG. PER IND.	
																								grains
1	A	1	2	1	1	5	5	1	2	1	2	1	0	0	0	1	0	3	2	3	1	32	1.6	
	B	1	2	6	3	7	4	4	7	5	1	2	0	2	3	1	4	3	3	1	3	62	3.1	
SAME YOLA SUSPENSIONS USED																								
2	A	2	4	2	1	1	2	1	5	5	3	0	1	4	0	4	3	0	4	1	2	45	2.25	
	B	1	8	6	1	4	4	3	8	4	1	5	7	6	7	2	4	1	0	7	2	81	4.05	

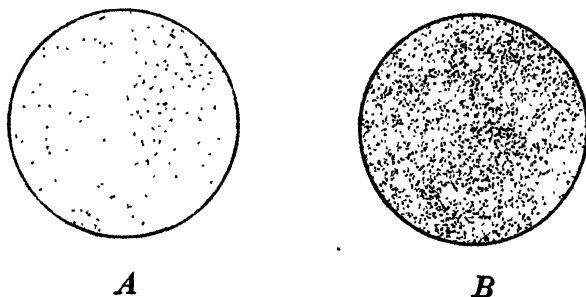


Fig. 3 Showing the relative concentration of yolk in dishes A and B of Experiment 1.

b. Effect of mechanical stimulation and of mechanical injury on feeding.

Experiment II. Thirty individuals from the same culture, starved twenty-four hours, were placed in each of six 8 cc. dishes containing 5 cc. tap water. Before feeding, the animals in three of the dishes (Set 1 in the experiment) were mechanically stimulated by means of a pipette. The opening of the latter was about ten times the width of Bursaria. The edges of the opening were made smooth by melting. The animals of Set 1 were stimulated by drawing them up into the pipette along with the tap water in the 8 cc. dishes, four times. Equal quantities of yolk suspension were now added to all the dishes. After having fed ten minutes the animals of Set 1 were again stimulated by drawing them along with the yolk suspension into the pipette two times; at the same time the control, Set 2, was stirred by gently shaking the dish and not allowing any instrument to touch the animals; hence the distribution of the yolk was the same in the two sets of dishes. All the individuals in Set 1, after having been stimulated, were perfectly normal and not injured. They looked like those of the

TABLE 5
Experiment II
SET 1. STIMULATED

DISH	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL	AVG. PER IND.	
																						grains	
A	0	1	4	0	1	0	1	3	1	5	1	1	2	0	1	1	0	1	1	1	0	33	1.1
B	1	0	0	2	0	0	0	0	2	0	2	0	0	0	2	0	0	3	0	0	1	17	0.56
C	4	0	0	1	2	1	2	2	6	0	1	1	1	3	3	1	1	1	1	3	0	48	1.60
Total average																						1.06	

SET 2: CONTROL: NOT STIMULATED

DISH	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																							TOTAL	AVG. PER IND.								
																									grains								
A	3	5	7	2	3	6	3	3	3	4	14	13	3	1	2	5	8	2	9	5	9	5	10	3	7	7	6	3	4	2	157	5.23	
B	2	1	3	6	3	1	5	1	3	6	4	4	5	2	3	2	5	6	11	4	2	6	6	1	3	2	7	2	3	0	109	3.63	
C	6	1	3	6	5	6	5	3	2	7	4	4	4	3	3	6	5	4	3	2	6	1	3	1	2	3	5	6	2	2	1	113	3.76
Total average																									4.208								

control. If a smaller pipette is used or a larger one, and the stimulation, by sucking them along with the medium up into the pipette, is more violent, it will stimulate and injure the organisms so that they will not eat at all, or at least, not for some time after stimulation. Of course structural injuries are very easily produced, with the result that regulation of the cell must take place before any food can be eaten (table 5).

Proof that in this experiment, Set 1, if not in Set 1 of Experiment III, the organisms were not injured beyond the capacity for swallowing, is found in the fact that the great majority did eat, though only a comparatively small number of grains. Another experiment may be given to illustrate the same fact.

Experiment III. The animals in Set 1 were not stimulated before feeding, but after they had fed for five minutes they were stimulated by drawing the suspension with the animals in it, up into the pipette only once. Material from a different culture was used in this experiment; time of feeding fifteen minutes. The control suspension with the organisms was redistributed once by gently shaking the dish. The animals were all normal in form at the end of the experiment (table 6).

In Experiment III the stimulus was only slight as compared to that in Experiment II, yet the effect was marked. As stated above, strong stimulation may totally prevent feeding.

TABLE 6
Experiment III
SET 1: STIMULATED

DISH	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																												TOTAL	AVG. PER IND.		
A	5	4	1	6	8	6	9	0	7	10	3	0	1	3	3	4	0	1	4	6	5	1	5	0	0	4	0	0	96	3.2		
B	0	0	4	0	4	15	11	0	0	9	0	1	4	6	5	2	5	8	4	0	9	6	6	11	4	5	6	0	2	0	127	4.0
C	5	3	0	4	0	2	1	3	5	0	3	1	0	1	0	4	2	0	3	1	3	3	0	8	0	0	3	0	4	59	1.96	
Total average																													3.5			

SET 2: CONTROL: NOT STIMULATED

[illegible]

The effect of mechanical stimulation must be emphasized because it shows that in any work of this nature it is necessary to handle the organisms gently. This relation must be inferred to apply to work on other Infusoria also, at least to some extent.

c. Effect of temperature on feeding.

Experiment IV. Thirty individuals starved twenty-four hours, were placed in each of six vials. Each vial contained 5 cc. of tap water. These vials were now placed in large dishes containing water kept at the desired temperatures. The latter were read on a small thermometer set inside of each vial. Equal quantities of fresh yolk suspension were added when the temperature had reached the desired point. They were fed fifteen minutes (table 7).

TABLE 7
Experiment IV

[illegible]

39 } All died in from 5 to 10 minutes
to }
40 }

The experiment was repeated with closely similar results. At lower temperatures the animals are always unable to eat. As the temperature is raised and the activity of the cell increases, the rate of feeding increases, continuing to increase nearly up to the point where the cell is injured or killed by the heat. At temperatures between 20° and 25°C . (i. e., at about the optimum) the increase in the rate of feeding can be determined only by using a very large number of individuals, since the variations obliterate the effects when a small number is used.

All the experiments relating to other conditions were carried on at temperatures ranging between 20° and 27°C . Where necessary (as in prolonged experiments on digestion) the temperature was kept constant to within 1° to 1.5°C ., throughout the course of the experiment, by keeping the organisms in moist chambers in a constant temperature oven.

d. Effect of HCl and NaOH on the feeding reaction.

Experiment V. The medium used in this experiment (table 8) was conductivity water.⁷ Any water less carefully purified is worthless for such experiments, as was shown by experiments carried out with tap water. By comparing the results it was strikingly evident that the acid and base had reacted with the salts and other impurities in the tap water and hence their effect was removed in low concentrations. The animals were washed once in conductivity water before putting them into the solutions. Time of feeding, twenty minutes (table 8).

It is seen from table 8 that the base NaOH was much more toxic than the HCl, and that as the concentration was increased the number of grains eaten became less and less. The chemical relations of the food and medium will be considered in more detail, later on (p. 29).

e. Effect of strong white light on the feeding reaction. Bursaria, when kept in dishes with a rather clear medium, often collect in the greatest number on the side of the dish away from fairly strong white light. It therefore became of interest to test what effect continuous light of a high intensity would have upon the rate of feeding.

⁷ Prepared and used in the Department of Physical Chemistry for conductivity measurements.

TABLE 8
Experiment V
NaOH

MOL. CONC.	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL	TOTAL NUMBER GRAINS
1/400.....	All dead in 3 minutes.....	0
1/600.....	All dead in 10 minutes.....	0
1/800.....	Many dead at 15 minutes; none eaten at end of 20 minutes.....	0
1/1200.....	All alive and normal in shape at end of ex- periment; no grains of yolk eaten.....	0
1/1600.....	0 1 0 0 0 1 1 0 2 1 2 2 0 1 3 2 0 3 0 0	19
1/3200.....	6 8 8 7 3 5 1 3 1 9 5 5 10 9 6 11 4 7 5 8	121
1/6400.....	5 9 8 8 6 14 8 1 11 5 8 5 12 4 8 11 6 8 6 5	148
CONTROL IN CONDUCTIVITY WATER		
	7 9 10 9 9 4 5 10 10 6 6 12 4 6 5 6 17 8 8 4	155

HCl

1/400.....	All dead in 5 minutes.....	0
1/600.....	0 1 2 1 1 1 2 0 2 1 4 2 0 1 2 0 5 4 1 2	32
1/800.....	1 3 4 5 13 4 5 5 2 6 0 5 4 4 3 3 7 5 5 6	90
1/1200.....	6 8 1 8 7 8 6 0 6 3 8 3 1 5 4 1 1 2 11 1	90
1/1600.....	6 5 8 8 7 9 3 12 3 2 5 8 14 6 9 3 5 5 0 5	123
1/3200.....	8 7 15 5 11 13 8 9 0 2 4 1 3 8 2 10 5 5 6 8	130
1/6400.....	5 10 12 10 9 7 9 3 14 6 10 5 9 9 10 10 7 9 5 3	162
CONTROL IN CONDUCTIVITY WATER		
	12 12 4 13 0 11 14 5 6 2 0 9 7 8 2 11 3 11 3 4	137

Experiments VI and VII. White light from the arc of an Edinger apparatus was focused upon the stage so that a spot of light $1\frac{1}{2}$ inches in diameter, of a very high intensity, was obtained. The light was filtered through a layer of water 1.5 cm. in thickness. An 8 cc. stender dish containing thirty normal individuals was placed in the spot of light and the usual quantity of yolk suspension added. A control was kept in weak diffuse daylight. The animals were fed twenty minutes. The following results show that continuous action of intense white light on the animals does not have any effect upon the rate of feeding. Two experiments with controls are given (tables 9 and 10).

TABLE 9
Experiment VI

STRONG WHITE LIGHT

NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																												TOTAL		
2	6	5	6	11	8	2	5	8	8	1	2	3	5	6	6	3	6	3	3	7	2	7	6	6	5	6	1	5	6	150

CONTROL: DIFFUSE DAYLIGHT

8	5	6	4	3	6	0	10	0	5	4	4	1	2	4	3	3	7	3	6	7	7	8	6	7	4	7	9	2	5	146
---	---	---	---	---	---	---	----	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-----

TABLE 10
Experiment VII

STRONG WHITE LIGHT

NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																										TOTAL				
5	6	9	6	3	1	5	3	3	5	8	1	7	6	4	11	7	7	0	2	10	4	3	4	4	5	11	3	6	5	154

CONTROL: DIFFUSE DAYLIGHT

8	8	2	5	3	10	9	11	8	9	2	7	8	1	0	4	15	0	8	2	8	10	6	7	6	8	11	3	4	5	188
---	---	---	---	---	----	---	----	---	---	---	---	---	---	---	---	----	---	---	---	---	----	---	---	---	---	----	---	---	---	-----

f. Effect of the electric current. Weak induction currents have, within a limited time, no noticeable effect upon the feeding, as is shown by the following results from two separate experiments, VIII and IX. The total number of grains eaten by 20 individuals in each of two 8 cc. dishes is given in each experiment:⁸

Experiment VIII

Dish A—73, total number of grains eaten by 20 individuals

Dish B—69, total number of grains eaten by 20 individuals

Experiment IX

Dish A—65, total number of grains eaten by 20 individuals

Dish B—64, total number of grains eaten by 20 individuals

Control for Experiments VIII and IX; not stimulated by the current

Dish C—89, total number of grains eaten by 20 individuals

⁸ The apparatus was arranged in such a way as completely to prevent any effect of substances liberated at the electrodes, by inserting the electrodes in a physiological normal NaCl solution in each of two 8 cc. dishes and from these the circuit was closed through the other two 8 cc. dishes containing the animals, by means, of small Ω tube connections filled with tap water and plugged loosely with a wad of cotton.

When, however, a direct current is used of such strength that the organisms can be made to go to one side or the other by reversal of the current the effect becomes more or less apparent. Feeding can not be prolonged to twenty minutes with a strong direct current, for the organisms are easily injured. To obviate this, time of feeding was limited to five minutes. The animals were made to swim from one side to the other by frequent reversals of the current. In Experiment X, they were stimulated by frequent reversal of the direct current during the first minute of feeding and then left to feed four minutes more without stimulation. In Experiment XI they were stimulated in the same way during the whole period of feeding. Time of feeding, five minutes (tables 11 and 12).

TABLE 11

Experiment X

STIMULATED BY DIRECT CURRENT, 1 MINUTE

DISH	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL
																					grains
A	3	13	5	11	3	10	8	0	1	6	11	5	8	4	3	7	1	1	8	3	111
CONTROL: NO CURRENT																					
B.	7	6	4	6	3	8	10	7	7	6	8	5	0	4	3	3	9	4	10	8	118

TABLE 12

Experiment XI

STIMULATED BY DIRECT CURRENT, 5 MINUTES

DISH	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																			TOTAL	
																				grains	
A.....	1	3	4	6	2	3	10	6	5	10	5	0	0	2	3	8	7	1	3	11	90
CONTROL: No CURRENT																					
B.....	5	5	11	0	2	18	3	1	11	0	11	13	10	0	5	7	0	9	5	6	122

As the results show, feeding was not discontinued under these conditions of strong stimulation by the current, though the or-

ganisms show a somewhat smaller total number of grains eaten than in the controls, in the same length of time. The difference is, however, too small to have a clear significance.

The strength of current may be increased but usually feeding can never be totally inhibited unless the organisms are injured or killed immediately after the yolk has been added.

The preceding experiments show clearly the relation which exists within certain limits, between the feeding reaction of this organism and a simultaneous reaction to certain other types of stimulation. During stimulation with HCl and NaOH, and especially with high temperatures and the electric current, the notable fact is that the reaction to food is strongly persistent under wide ranges of intensity of a second applied stimulus; this is true to such an extent that under some conditions the feeding continues up to the point where the intensity is so high that the stimulus is destructive to the organism. These facts must not be thought to be of general application, for evidently mechanical stimulation is quite effective in changing the reaction to food. What the behavior will be under two simultaneous stimuli obviously depends upon the nature of those stimuli.

It should be distinctly noted that in all the foregoing experiments the chemical as well as physical nature of the food substance has been kept constant while the organism in its particular physiological state has been acted upon by certain external agents; these being of a sufficient variety to indicate clearly what rôle these different types of factors play in the relation of this animal to food, and to serve as a guide to further inquiry.

We now have to see what changes are produced in the feeding reaction by modifying that factor which in the foregoing experiments has been kept constant,⁹ namely, the food. In the following series of experiments all the other conditions will be kept constant, or at least arranged in such a way that they may be

⁹ An exception to this might be taken in the experiments with HCl and NaOH for it is a question whether or not these affect the chemical character of the yolk sufficiently under the conditions of these experiments to modify the number of grains eaten. The yolk was not treated previous to the feeding; thus the time was so short and the dilutions so high that any change must have been very slight.

properly controlled and accounted for. We shall attempt to determine what the relation of Bursaria is, to specific physical and chemical properties of the food itself. First it will be determined how the external part of the reaction is modified, that is, what is the behavior of the cell in so far as this has to do with the selection of food.

SELECTION OF FOOD AND THE FACTORS CONCERNED

The object of the experiments described in the present section is to answer the question: Can Bursaria discriminate quantitative or qualitative differences between the yolk grains?

When fresh hard boiled yolk grains, prepared as described on page 8, are treated with different kinds of water-soluble dyes, the amount of dye which is adsorbed by a grain of yolk varies with the kind of dye used. At first a considerable number of different dyes in aqueous solution were tested in a comparatively rough way; first, for the relative amount of each dye which would be taken up by the grains of yolk; second, for the rate at which the dyes were adsorbed and the ease with which they could be washed out (reversibility of the adsorption); and third, for the relative toxicity of aqueous solutions of these dyes to the organisms. Among the dyes so tested were fuchsin, lyons blue, methylin blue, eosin, cyanin, gentian violet, saffranin, janus green, congo red, and an aqueous solution of hematoxylin.

The results of the following experiments on food selection, in so far as they are related to the dye, depend upon the three factors named: (1) The amount of dye adsorbed (2) The rate of the reversible adsorption reaction, and (3) The relative toxicity to Bursaria, of the dye in aqueous solution.

It was quickly found that certain dyes were better suited than others, for the particular end in view. Aqueous solutions of saffranin and janus green were found best to fulfil the necessary conditions. Both show a reversible adsorption with yolk, while the velocity of the reversible adsorption is sufficiently low to prevent a too rapid washing out of the stain. By this means one is able to control the amount of adsorbed dye much more

easily than if it could be washed out quickly, and one is also able to control the concentration gradient between pure water and the dye adsorbed by the yolk grain. The toxicity of the different dyes varies greatly, and it was found that saffranin and janus green were best from this point of view also, since both of these are very toxic to *Bursaria* in higher concentrations but only slightly so in lower concentrations.

1. Experiments with stained and unstained yolk

a. Saffranin.

Experiment XII (a). Object, to test (a) whether or not *Bursaria* will eat yolk grains which have adsorbed an appreciable amount of the soluble toxic substance saffranin and (b) whether or not the amount of yolk eaten depends, in this experiment, upon the amount of saffranin adsorbed.

Equal volumes of a strong suspension of fresh yolk were placed in each of seven stender dishes of 8 cc. capacity. A bright rose-colored solution of saffranin was made up with tap water. To the dishes designated A, B, C, D, E and F was added 5, 4, 3, 2, 1, and 0.5 cc. respectively, of this solution, and mixed thoroughly. The seventh dish without stain, was kept as a control. The suspensions were left to settle five minutes, then decanted and 5 cc. tap water added to all the dishes; this was repeated three times. The organisms used were starved twenty-four hours and were in excellent condition. The time of feeding was fifteen minutes (table 13)

TABLE 13
Experiments XII (a)

DISH	SAFFRANIN SOL. C. C.	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL
A	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
B	4	0	0	0	0	4	8	0	3	1	0	0	0	0	1	3	0	1	0	0	0	34
C	3	3	0	6	0	0	2	0	8	3	1	2	8	4	2	4	0	1	0	0	2	61
D	2	2	0	0	3	1	1	0	2	4	2	1	2	2	1	3	3	2	0	6	5	56
E	1	4	3	1	2	7	6	2	1	7	9	0	3	7	2	0	12	6	4	0	4	113
F	1/2	4	6	11	6	1	2	4	0	3	0	3	9	3	4	3	6	5	4	5	9	138
Control																						
G	0	0	5	6	6	7	4	9	12	4	9	9	11	4	2	7	7	9	6	12	1	188

When stronger solutions of saffranin than that in A were used, no grains were eaten. All the animals at the end of the experiment were normal and had not been injured. The yolk of dish

A was now left to soak in its water for fifteen minutes longer, this water was then drawn off and the yolk again washed twice with water. Thirty individuals from the same material as used above were now put into the dish. At the end of fifteen minutes the following was the count:

Experiment XII (b)

3, 1, 5, 1, 3, 4, 4, 3, 9, 3, 7, 3, 0, 4, 5, 3, 6, 0, 0 4, 1, 4, 5, 0, 0, 2, 3, 1, 3, 1. Total 88 grs.

This indicates that we may obtain the same result whether we proceed with a strongly stained yolk and test successively after each washing, or, as in the former experiment, by staining different portions of yolk to different degrees to begin with. This was actually done in other experiments not given, and results exactly similar to those in Experiment XII (a) were obtained.

To show that even a considerably stronger medium does not injure the animals seriously, a yolk suspension stained with saffranin more strongly than that used in A of Experiment XII (a), was made by leaving the yolk several hours in a very strong solution of the stain. This was washed out several times and then thirty individuals from the same culture material used in the former experiments were put into it and left for fifteen minutes. They were then picked out and washed once in tap water, and then transferred to an unstained yolk suspension for fifteen minutes. The count gave the following (table 14):

TABLE 14
Experiment XIII

MIXTURE.	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																	TOTAL
Stained ..	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Unstained .	0	2	0	3	0	0	0	0	1	0	3	1	0	0	2	1	3	35

This shows that they were not injured sufficiently in even this strongly stained suspension totally to prevent them from eating unstained yolk immediately afterward. The cause of most of the 0's in the count is that, as the toxicity of the solution increases, the organisms have a tendency to close up the oral apparatus and do not open it again sufficiently, within the next twenty minutes or so, to be able to take in the yolk grains. Of

course if yolk which has been very strongly stained and not washed out sufficiently, is fed, then the concentration of the medium rises so quickly that they are greatly injured or killed.

Now against the conclusions which will be drawn from the results of Experiment XII (a) and (b), as it stands, may still be urged the objection that the reason that so few or no grains are eaten, is because of what one might call a general injury or stimulation of the cell by the saffranin which is rapidly being liberated into the water, and that it may not have anything to do with a specific reaction to the chemical character of the food particle as such, that is, to anything like a "sense of taste." That this objection does not apply to conditions like those in the above experiment (XII, a and b) where the amount of stain adsorbed even in dishes A and B is very little compared to that in Experiment XIII, may be shown by taking the solution of dish A, Experiment XII (a), and placing unstained yolk and *Bursariae* in it. The result of such an experiment is that the organisms fill up with fresh yolk, showing that the medium in weaker concentrations does not affect the eating process to any appreciable extent. Further proof of this will be given in the experiments immediately following, and also in experiments to be given later.

Experiment XIV. To test whether or not *Bursaria* can select and eat non-toxic yolk grains from among toxic ones, when the two are mixed. Two suspensions were made, one of yolk stained in saffranin twenty-four hours, then washed out repeatedly, the other containing the same kind of yolk washed in the same way but not stained. The two yolk suspensions were mixed immediately before the animals were placed in the mixture. Twenty individuals were used. The time of feeding was fifteen minutes; a control of washed unstained yolk alone, was kept at the same time (table 15).

TABLE 15
Experiment XIV

MIXTURE	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL
Stained.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unstained.....	0	5	2	3	2	1	2	4	2	0	1	2	3	2	0	0	0	0	0	0	29
Control:																					
Unstained yolk.....	12	16	7	15	4	9	15	8	9	9	16	3	25	7	15	12	9	9	5	8	213

In the mixture the concentration of the saffranin rose so rapidly that some of the individuals were killed.¹⁰ This is indicated above by X. Yet even in such a strong solution, selection took place, though the number of grains eaten was small compared to the number in the control.

Experiment XV. Another sample of yolk less deeply stained than that in Experiment XIV, was washed out many times and mixed with an equal quantity of unstained yolk from the same sample. Thirty individuals of the same material as used in Experiment XIV, were fed for five minutes, instead of fifteen minutes as before (table 16).

TABLE 16
Experiment XV

MIXTURE	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																												TOTAL		
Stained.....	1	1	1	1	0	1	1	0	3	3	2	2	0	1	0	3	1	2	4	4	1	2	2	1	2	1	0	0	1	2	43
Unstained.....	2	6	7	2	16	14	4	11	11	12	9	7	13	9	0	6	5	12	9	5	7	2	10	7	5	7	3	8	6	216	

A repetition of the above experiment with yolk stained a little more deeply gave the following result; twenty individuals used; fed five minutes (table 17).

TABLE 17

MIXTURE	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL
Stained.....	0	0	3	1	2	2	1	0	1	1	1	2	1	3	0	1	3	2	1	0	25
Unstained.....	4	0	0	1	4	5	5	0	4	2	3	5	3	0	1	3	0	12	1	3	56

The control of Experiment XIV will likewise serve for Experiment XV.

The results of this experiment are to be explained by the fact that the concentration gradient of the adsorbed toxic saffranin is relatively low with respect to the gradient of the water-soluble yolk substance to which *Bursaria* reacts in a strongly positive manner. Of course one is not to suppose that it is the relative molecular concentration gradient alone that determines the re-

¹⁰ The cytolytic action of saffranin is in some ways more marked than that of janus green. The character of its reversible adsorption reaction also makes it less suited for use in experiments of this kind than janus green, as will appear from results with the latter.

sult. What is meant in this case by concentration gradient, is the molecular concentration plus the specificity of the substance, that is, in anthropomorphic terms we should say "the kind of taste" which the substance has. That the specific nature of the substance is one factor in determining the result, is shown by a comparison of the results of numerous experiments with saffranin, janus green, hematoxylin, and especially other less toxic stains, like congo red (cf. what follows).

b. *Janus green*. A considerable number of experiments have been carried out using this substance, with the same general results as those obtained with saffranin. It is better adapted to bring out the phenomenon of selection than saffranin, causing a sharp discrimination by *Bursaria*; small quantities adsorbed by the grains are sufficient to bring about rejection. The following experiments show some of the relations.

Experiment XVI (a) and (b). Yolk was stained in janus green twenty-four hours then soaked in tap water and washed repeatedly. A portion of the same kind of yolk soaked and washed in the same way but not stained, was used as a control and for mixing with the stained yolk. A few minutes before the experiment equal quantities of the stained and unstained yolk suspension were mixed in dish A. A second quantity of the unstained yolk suspension of a concentration equal to the sum of those in dish A was placed in dish B. Twenty individuals were placed in each dish and left to feed twenty minutes (table 18 a).

TABLE 18 (a)
Experiment XVI (a)

MIXTURE, DISH A	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL
Stained.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unstained	5	3	1	6	2	4	1	4	5	1	5	1	4	4	1	1	1	1	1	1	1
Control,																					
Dish B:																					
Unstained.....	9	12	21	8	20	12	15	19	13	17	14	13	15	12	19	17	14	7	8	3	268

The yolk in both dishes was now washed twice and the experiment repeated with control. Time of feeding fifteen minutes. The count is shown in table 18 b.

In (a) the solution had become sufficiently strong to affect five of the animals (X), so that they could not be recovered for

TABLE 18 (b)
Experiment XVI (b)

MIXTURE, DISH A	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL
Stained.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unstained.....	0	0	0	1	0	0	1	1	2	0	1	2	2	4	0	0	1	0	1	3	19
Control,																					
Dish B:																					
Unstained.	16	8	17	7	13	10	11	12	5	12	4	5	12	15	15	16	7	10	13	8	216

the count. The smaller number of grains eaten in (b) by those in dish A is due in part to the shorter time of feeding but more to the fact that the unstained yolk grains had by this time adsorbed some of the liberated janus green from the stained yolk grains (see Experiment XVIII, p. 36).

Experiment XVII (a) and (b). The results of this experiment are given to show that Bursariae from two different cultures may show different reactions in selection experiments. In part (a) material was used from one wild culture, while in part (b) material from a different one was used. Both were starved twenty-four hours before using them. All other conditions were alike. Time of feeding fifteen minutes (table 19).

TABLE 19
Experiment XVII

MIXTURE, DISH A	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL
Stained.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unstained.....	0	4	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	9
Control: dish B,																					(a)
Unstained.....	5	4	6	2	4	7	6	3	9	3	8	3	3	6	4	3	2	7	6	8	99
Stained..	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unstained.....	3	4	0	2	10	3	3	2	7	6	1	6	6	0	8	2	1	0	5	1	70
Control: dish B,																					(b)
Unstained.....	2	4	13	9	5	7	9	2	4	10	6	15	9	13	6	11	9	1	6	7	148

In such experiments as these it was found that occasionally an individual had eaten a stained grain along with the unstained ones, but this happened very seldom in any of the experiments with janus green.

cially in higher concentration, are very injurious, the organisms, nevertheless, eat the grains of yolk. After one or more hours the grains become stained deeply. This was the case in table 22 (b). The increase in the length of time of feeding (i.e., the time the animals were left in the solution) is the cause of the high mortality in table 22 (b).

TABLE 22 (a)
Experiment XX

NUMBER OF DISH	1	2	3	4	5	6	7	8	9
Number of drops of $\frac{1}{2}$ per cent aq. hema- toxylin	4 0 4 6 2 2 0 4 6 0 4	8 0 1 2 5 4 0 1 7 3 0 0	12 0 2 0 4 0 0 1 3 0 7 0	16 2 3 11 1 1 2 4 4 13 ×	20 lost 	24 1 4 7 3 1 11 7 0 2 4	28 4 6 3 7 1 1 4 4 2 ×	50 7 0 ×	0 7 6 10 11 13 8 14 6 6 8
Number of grains eaten by each in- dividual									
Total.	28	23	17	38+		40	32+	(7+)	89

TABLE 22 (b)

NUMBER OF DISH	1	2	3	4	5	6	7	8	9
Number of drops of $\frac{1}{2}$ per cent aq. hema- toxylin.	4 3 5 0 3 2 5 3 3 3	8 1 1 2 3 0 6 0 3 ×	12 0 1 0 4 0 0 0 0 ×	16 1 2 1 0 ×	20 1 ×	24 0 3 0 2 ×	28 ×	50 ×	0 11 8 7 11 14 8 6 8 13 7
Number of grains eaten by each in- dividual									
Total	33	17+	8+	4+	1+	5+	—	—	93

That the yolk grains are eaten, though to a less extent, even after having been left in the solution of 50 drop concentration for three and one-half hours, is shown by the following: Feeding was limited to three minutes, which in part accounts for the comparatively small number of grains eaten. Ten individuals were used. The count gave 4, 2, 1, 6, 1, 1, 2, 0, 0, 1, a total of 18 grains of the deeply stained yolk. When this deeply stained yolk in the 50 drop concentration of hematoxylin was washed four times in tap water and tested again; the following count was made: 11, 13, 14, 6, 5, 2, 12, 2, 4, 5, a total of 74 grains.

These tests show (a) that although a dye may be toxic to Bursaria, it may nevertheless not affect, to any great extent, the functioning of the feeding mechanism in the taking in and swallowing of the food, though (b) with some dyes total rejection of the food may take place, when the concentration is so low that it has only a comparatively slight cytolytic effect. The former condition is shown to a less marked extent in the experiments with saffranin than in the experiments with hematoxylin; while the latter condition is illustrated by the results with janus green. This seems then also to strongly suggest that different substances may affect different parts of the cell differently. Corroborative evidence upon this point, which it would be out of place to consider here, has been obtained from observations showing that the localization of the beginnings of cytolysis of the cell body of Bursaria may differ with the particular nature of the toxic agent employed.

d. Congo red. Another stain which is adsorbed readily is congo red. This, however, unlike hematoxylin, can only be washed out in part, that is, its adsorption reaction is not completely reversible. Also since this dye is not as toxic as saffranin or janus green, a large quantity of the stain may be adsorbed and yet not appreciably affect the number of grains eaten, as is shown by the following experiment.

Experiment XXI. The yolk was stained twenty minutes in a strong aqueous solution of the dye. Time of feeding twenty minutes. Thirty were used (table 23).

TABLE 23
Experiment XXI

	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																												TOTAL		
Congo red, dish A																															
Stained	7	3	6	1	7	6	2	5	1	2	5	4	4	3	3	2	4	4	4	0	5	1	7	3	2	4	6	5	5	4	115
Control dish B																															
Unstained	11	8	7	3	6	8	5	6	6	5	8	7	5	1	2	5	6	7	1	5	9	6	7	10	5	6	5	9	4	7	179

In this case we have a comparatively low concentration gradient of the dye, together with a low toxicity and hence the comparatively small difference in the readiness with which *Bursaria* eats the stained and unstained yolk.

e. Sudan III. To show that an adsorbed substance which is insoluble in the medium has no determining effect upon the feeding and food selection, Sudan III was employed. This substance is insoluble in water but soluble in ethyl alcohol and fats.

Experiment XXII. Fresh yolk was stained in an 80 per cent alcoholic solution of Sudan III for a short time. It was then dried in an oven at 27°C. for twenty-four hours. A control of fresh yolk was also kept. The organisms were fed twenty minutes. The yolk takes on a very deep color with this stain.

TABLE 24
Experiment XXII

	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																				TOTAL
Sudan III, stained yolk	13	18	19	0	15	3	5	7	18	25	15	5	7	8	9	17	18	12	3	26	243
Control, unstained	18	3	4	14	7	15	17	22	6	28	20	6	9	3	29	18	13	10	8	24	274

It is evident that the insoluble Sudan III had no appreciable effect upon the food reaction. Mixtures of these showed no difference in the amounts of the two kinds of yolk eaten.

f. Stale yolk.

Experiment XXIII. Mixtures of fresh and stale yolk could not be used since the grains of the two kinds of yolk were visibly indistinguishable. One experiment is given. The stale yolk was four weeks old while the control was freshly prepared; both were of the same concentration (table 25).

TABLE 25

Experiment XXIII

	NUMBER OF GRAINS EATEN BY EACH INDIVIDUAL																TOTAL	
Stale yolk.....	0	2	5	1	0	3	8	2	1	1	4	0	6	1	5	1	3	48
Control, fresh yolk..	8	4	7	2	1	1	4	4	9	3	0	7	9	5	2	2	6	96

A difference is plainly evident. Other experiments show greater or less difference, depending upon the conditions.

2. The basis for and nature of food selection in Bursaria, as shown by the foregoing and other experiments

It must be remembered that in any such experiments as the foregoing the relation to food is in some ways an entirely new one to the organism. Yet it must be insisted upon that the yolk used in these experiments is assimilable by the organisms (a fact which will be considered at length in a later paper) and especially that whatever the mechanism of feeding and selection in nature is, it must be the same one which is brought into action in these experiments. Hence the criticism imagined above would appear to have no importance for the question under consideration here. In fact, it is to be believed that so far as these experiments are concerned, they are only a more strongly emphasized condition of what we find in nature and that they picture to us, so far as they go, the actual condition of the food relation of *Bursaria* in its native culture.

We may state the results briefly in the following way: First: Yolk grains are rejected if the soluble adsorbed toxic substance makes with the medium a sufficiently steep concentration gradient. If this gradient is low relative to that of the yolk-soluble substance, to which *Bursaria* reacts positively, then the organism may eat the stained yolk, other conditions being equal. Second: (a) Whether *Bursaria* will eat stained yolk grains or reject them depends also, along with the steepness of the concentration gradient, upon the specific chemical properties of the adsorbed sub-

stance in question, and furthermore (b) the substance by virtue of its chemical properties has, at least in some cases, a specific action upon the mechanism of feeding and selection, as is shown by a comparison of the results of the experiments with hematoxylin, saffranin and janus green. Additional evidence obtained from observations upon the phenomena of cytolysis in *Bursaria* also points to the correctness of this conclusion. A familiar instance of a similar nature is the casting off of the peristome by *Stentor* when stimulated or injured by chemicals. Another instance is the fact found by Jennings that the anterior end in *Paramecium* is more sensitive to mechanical stimulation than are other parts of the body.

That in feeding experiments with the Protozoa it is difficult to discriminate closely between the effects of the medium and those of the food substance itself is obvious, since (a) the amount eaten depends upon so many factors other than the nature of food and (b), since the organism selects on a chemical basis, which involves a soluble substance or substances diffusing into the medium from the food particle, hence necessarily involving the external medium to a greater or less extent. It is of course clear that differences in certain physical characters of food may likewise determine whether or not it will be eaten. This is shown most simply by objects which are too large, such as large yolk grains and large individuals of *Stentor*, which cannot be swallowed.

From all the facts found from experiments upon food selection by *Bursaria*, there is no evidence that active selection is based upon either "size, weight, form or surface texture" or any combination of these, except in so far as simple mechanical conditions would make them effective. All the facts show clearly that the chemical nature of the food is the property upon which the power of discrimination by *Bursaria* depends. Hence I find no evidence from *Bursaria* to support Schaeffer's contention that "*Stentor* selects its food upon a tactual basis and apparently not upon a chemical one" and that "*Stentor* reacts in selecting food, to physical properties only or chiefly, and not to chemical properties" (Schaeffer '10, page 131). On the other hand, the

facts which have been found in this connection are in agreement with the results and conclusions so far as they have been worked out by Metalnikow ('12) for *Paramecium*.

THE RELATION OF BURSARIA TO DIGESTIBLE AND NON-DIGESTIBLE SUBSTANCES

1. The external relations

Many substances which are in the ordinary sense chemically indifferent to the organism are likewise eaten, though generally in small quantities. Among these are cinnabar, carbon black, chinese ink, powdered aluminium and the like. The relation of Bursaria to this class of substances is however strikingly different, inside of the cell and to a large extent outside, when compared to that relation in the case of digestible and assimilable ones. The fact that some comparatively indifferent substances like the above, are eaten does not affect our conclusion drawn above, as to the paramount importance of the chemical properties of the food in food selection. Chinese ink contains some mucilaginous matter which as my own observations have shown me, is reacted to positively by Bursaria and hence the ink is quite readily eaten. Carmine is a similar substance which though generally taken to be insoluble in water, is in fact sufficiently soluble clearly to affect the feeding reactions of Bursaria. Furthermore, the fact that a substance may be insoluble does not, of course, prove that the stimulus from it is not a chemical one, for it is probable, that with such substances as aluminium, catalytic or other specific chemical, or even physical reactions dependent upon the chemical properties of the substance, are produced by contact with the plasma membranes. The possible variety of interactions of the cell with different kinds of substances when considered in this order of magnitude may of course be very large.

As regards the eating of non-digestible substances, powdered aluminium may serve, in one way, to illustrate the external relations. If a large number of individuals are put into a suspension of aluminium, often few if any will eat any of the particles of

aluminium and those that do eat it generally take in only a small quantity. This is also true of Sudan III and of carbon black. The quantity eaten varies with the conditions in a similar way, as previously set forth for yolk. Now if fresh yolk grains are added to the suspension of aluminium the animals will often quickly fill up with yolk, but in this case flakes of aluminium become attached to the yolk particles and hence often considerable quantities of the metallic aluminium are passed into the body along with the yolk. Sometimes the quantity of yolk eaten in such a mixed suspension is less than that in the control. This serves to illustrate the sort of equilibrium which exists between the organism and the kinds of substances in suspension, partly determining the amount of food and other substances eaten.

2. The internal relations

It was interesting to find that *Bursaria* possesses what I shall call an internal compensating reaction to those substances which are eaten to some extent, but are not digestible, such as Sudan III, chinese ink, powdered aluminium, and so forth. This compensating reaction makes up to some extent, in the "economy of the organism" for the lack of a perfect discrimination between indigestible ('tasteless') substances and those which can serve as food. It is shown by the fact that indigestible substances are eliminated from the cell usually a long time before the digestion of a similar quantity of food is completed. This may be shown with Sudan III. The results of the experiments are, for the sake of brevity, given by curves.

Experiment XXIV: Figure 4. Three sets of twenty-four individuals each were fed Sudan III, cold-ether-extracted yolk, and fresh yolk respectively. They were placed two in each watchglass containing tap water in moist chambers, and examination in this case was made at the end of three, seven, and twenty-two hours. Points on the abscissae indicate the length of time in hours after feeding, while points on the ordinates show the number of individuals which had extruded Sudan III (curve *A*) in the time intervals between the examinations, or in the case of extracted yolk (curve *B*) and fresh yolk (curve *C*) the number of individuals that had lost all traces of food. In this experiment the observations were not sufficiently frequent to bring out the actual

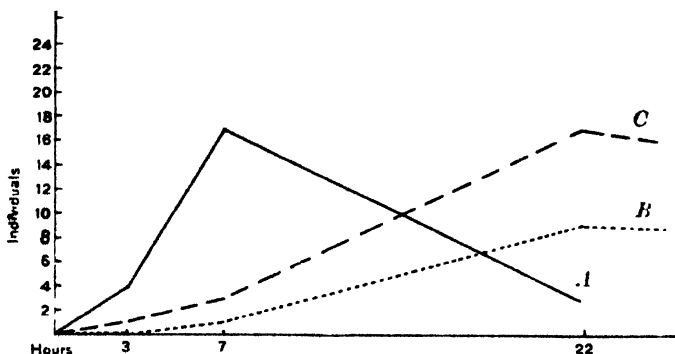


Fig. 4 Experiment XXIV. Curve A represents the course of total extrusion of Sudan III; curve B, that of complete digestion of cold-ether extracted yolk; curve C, course of complete disappearance of fresh fat-containing yolk.

course of the extrusion of Sudan III or of the disappearance of the yolk from the cytoplasm, but it will be noted that the great difference appears in the observation at the end of seven hours. At the end of twenty-two hours A had no traces of Sudan III, B still had six and C had ten individuals with food.

This early extrusion of indigestible substances is considered in more detail in connection with chinese ink in the following.¹¹

Experiment XXV. Two sets of forty-eight individuals each were fed, one with cold-ether-extracted yolk, the other with chinese ink. Examination of the cell content was made at hour intervals as indicated by numbers on the basal abscissa. Ordinates indicate the number of individuals which had extruded all the ink content in the time indicated (curve A). In curve B the ordinates represent the number of individuals fed extracted yolk in which yolk had disappeared at the end of the time indicated by the abscissa. Chinese ink in suspension is much more readily eaten than carbon or aluminium and is therefore more convenient. This is to be explained by the fact that there are present mucilaginous soluble substances in the chinese ink which serve as agents inducing a more positive feeding reaction and are possibly of some slight food value. The ink was not found to be injurious to the animals. The greater part of the ink is thrown out quite early while slight traces may remain for some time longer. The time during which the ink was retained was taken to end when the last trace

¹¹ In order to obtain satisfactory results with such substances as Sudan III and aluminium in aqueous suspension the adsorbed gases should be driven off before feeding.

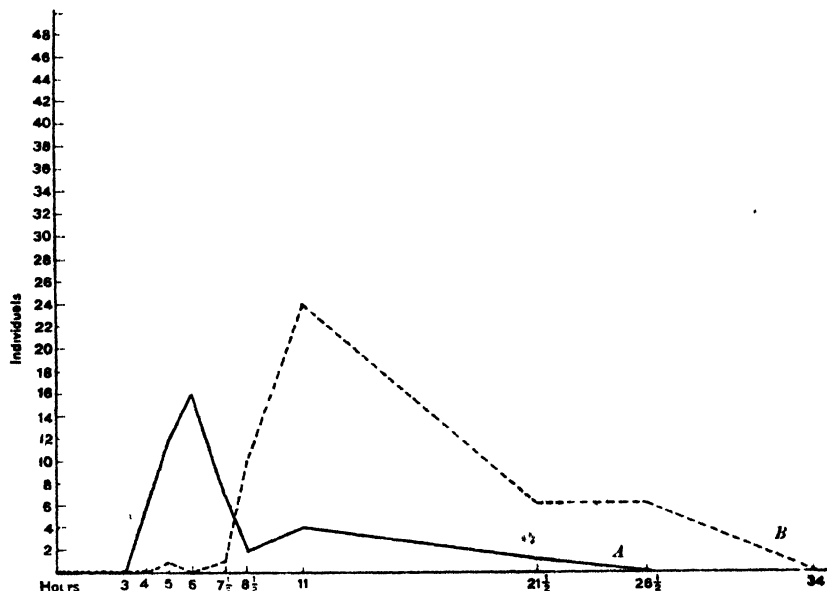


Fig. 5. Experiment XXV. Curve A represents the course of extrusion of Chinese ink by forty-eight individuals; curve B that of complete digestion of a similar quantity of cold-ether extracted yolk by another set of forty-eight individuals from the same culture.

had been eliminated; curve A does not therefore represent the actual time at which the greater part of the ink was extruded but should have its maxima farther to the left than shown. This statement applies to all the extrusion curves which are given.

In figure 5 curve B, is that of complete digestion; no extrusion of the extracted yolk took place in this experiment.

After it had been thus shown that ink fed alone to one set of individuals was extruded long before digestion is completed of a similar amount of extracted yolk fed to another set, experiments were carried out to test what the reaction would be if both ink and extracted yolk were fed to the same individuals at the same time. The following two experiments are given to bring out the facts in a quantitative way.

Experiment XXVI. Fifty-four individuals were first fed Chinese ink and immediately afterwards fed with cold-ether-extracted yolk. Two individuals were placed in each watch-glass containing 5 cc. of tap

water and kept in moist chambers. Records were taken noting the presence or total absence of ink and presence or completion of digestion of the extracted yolk at one hour intervals beginning with three and one-half hours up to twelve hours after feeding; three more records were taken at twenty-four, thirty-three and forty-eight hours. The results are expressed in curves in figure 6. Curve *A* represents the extrusion of ink; curve *B*, that of complete digestion of yolk.

It is seen from the relation of the curves that even in this case the ink is extruded before digestion of the extracted yolk is complete, provided that a sufficient quantity of yolk has been eaten.

It was noted that a short time after the ink had been eaten it became assembled into one or several rather definite lumps. This takes place before extrusion. Closer observation further revealed the fact that when ink particles came to be included in vacuoles containing yolk they were not extruded until the food of those vacuoles had been digested, while those which were not included by the yolk vacuoles were very soon extruded. This fact can readily be made out while one follows such experiments as Experiment XXVI above. Bursaria therefore has a power of simultaneous selective extrusion of the contents of different vacuoles as well as a power of selection in the feeding process. This mechanism obviously compensates for the lack of a perfect discriminative and selective function of the oral apparatus.

The results of an experiment (fig. 7) where these facts were taken into account for the purpose of expressing them in a graphic way in curves, is given in the following experiment. A control for comparison was also kept in this case (fig. 7, curve *C*).

Experiment XXVII. Forty-eight individuals were used in each of both the experiment and control. The control (curve *C*, fig. 7) which was fed ink only, shows a sharp early maximum of extrusion from five and one-half to seven and one-half hours after feeding, with three or four individuals retaining traces of ink as long as ten and one-half to twelve hours. Curve *A* represents the extrusion of ink in the forty-eight individuals fed both ink and extracted yolk. It shows two maxima exactly similar to those of curve *A* in Experiment XXVI. Curve *B* (fig. 7) represents the course of complete digestion of the yolk in the same individuals as those of curve *A*. There is only one maximum

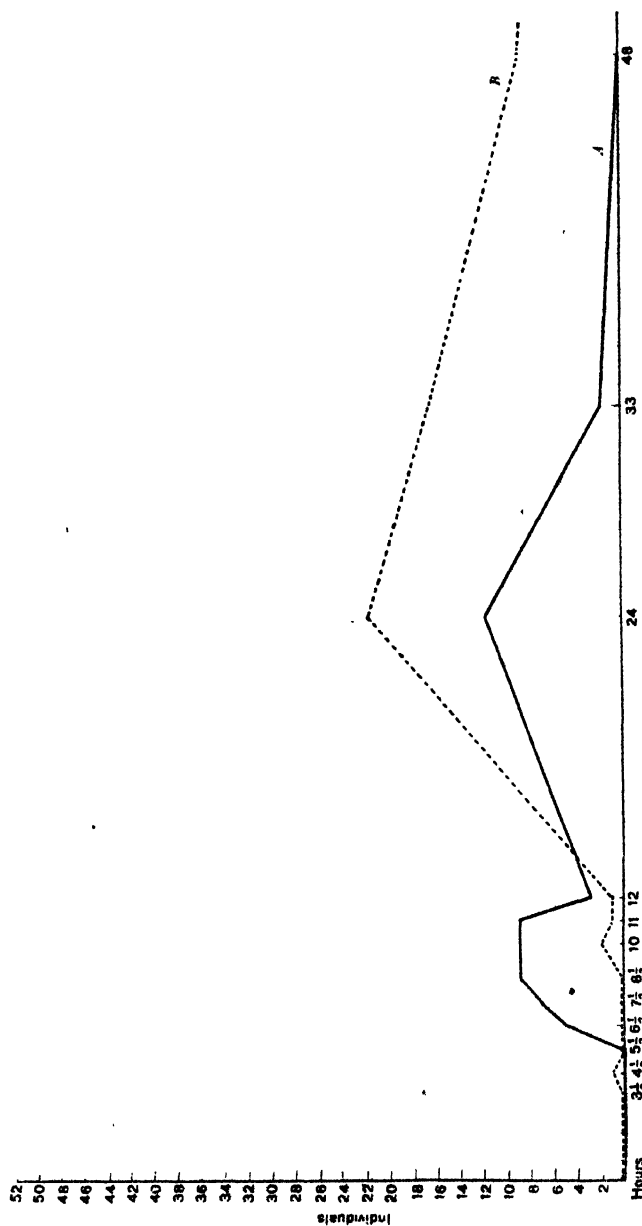


Fig. 6 Experiment XXVI. Curve A represents the course of complete extrusion of Chinese ink fed to fifty-four individuals; it shows two maxima; curve B, course of complete digestion by the same individuals as of curve A; curve B has only one maximum.

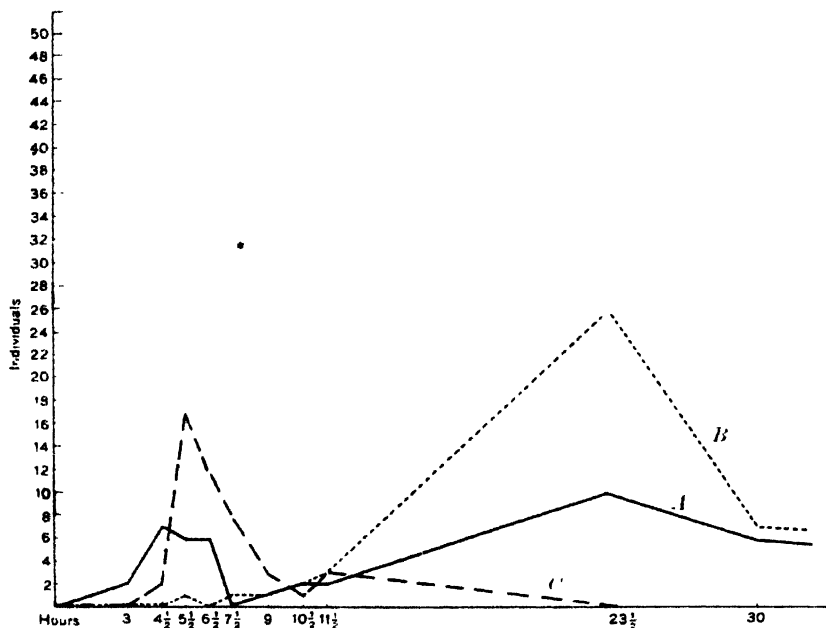


Fig. 7 Experiment XXVII. Curve A, course of extrusion of Chinese ink showing two maxima (cf. fig. 6, curve A); curve B, that of complete digestion, by the same individuals used in curve A, of extracted yolk; curve C, control: course of extrusion of ink by forty-eight individuals fed ink alone.

in this curve, and this comes about eighteen hours later than the first maximum of curve A and at about the same time as the second maximum of B. The significance of the second maximum in curve A is brought out in the following analysis of the two curves A and B, that is, in a quantitative analysis of the reactions of the forty-eight individuals used in the experiment. All the individuals which at any of the examinations had ink and yolk present in the same vacuole, or vacuoles, were recorded, hence we have a means of dividing the forty-eight individuals into two groups. Group I is made up of those in which, throughout the experiment, yolk and ink were in distinct and separate vacuoles, while Group II, includes those which had during part or all of the time one or more vacuoles which contained both ink and yolk in the same vacuole or vacuoles. Now we have the data which will show just what relation ink and yolk have to each other in the cytoplasm of Bursaria, and what the reaction of the cell is toward each of the two conditions represented by the composition of the vacuoles of the two Groups I and II.

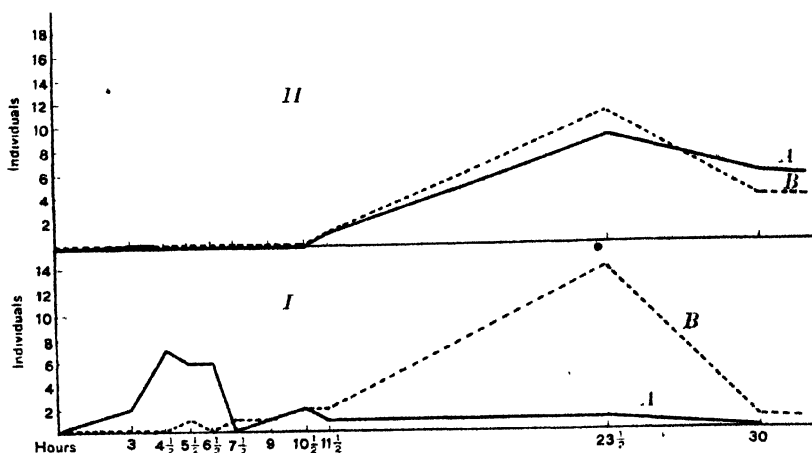


Fig. 8 Analysis of curves A and B of Experiment XXVII, figure 7. *I*, curve A, course of complete extrusion of ink from vacuoles containing only ink; curve B, course of complete digestion of extracted yolk in vacuoles containing only yolk, of the same individuals used in curve A. *II*, Curve A, course of complete extrusion of ink from vacuoles containing both ink and extracted yolk; curve B, course of complete digestion of extracted yolk from the same vacuoles in the same individuals as in curve A.

We may plot the curve of extrusion of ink in group I, and the curve of complete digestion of the same individuals. The curves are given in figure 8, *I*. The same was likewise done for Group II (fig. 8, *II*).

Curve A of figure 8, *I*, (ink and yolk in separate vacuoles) shows now only one early extrusion maximum instead of two. Curve B of figure 8, *I*, is lower (owing to the smaller number of individuals) but exactly similar to B of figure 7. The extrusion of ink from a cell which has its yolk and ink in separate vacuoles is therefore independent of the presence of food and occurs a long time before digestion of the food is completed. The curve of extrusion of ink, A, figure 8, *II*, shows now only one maximum and this corresponds to the second maximum of curve A, figure 7, and is practically identical with curve B of complete digestion. This shows then that whenever ink is included with food in the same vacuole it is retained until its accompanying

food is completely digested, because the maxima of the curves *A* and *B* of *II*, and of *B*, *I*, occur at the same time. We therefore have a demonstration of the selective extrusion among vacuoles as well as of a process of selection in feeding in *Bursaria*. Similar curves may be worked out for Sudan III or powdered aluminium.

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SUMMARY

1. *Bursaria* has three ways of rejecting solid particles, as shown by the paths over which the particles are passed. These are: (a) the path of total rejection, shown by particles which never enter the oral apparatus; (b) the path of rejection of large particles, this being a retracing in the opposite direction of the path by which they entered; (c) the path of rejection of small particles, which leave the oral pouch by way of the base of the oral sinus and are passed backward over the ventral side of the body (fig. 1).

2. No definite path is followed by the food vacuoles during digestion, and in their passage through the cytoplasm. Residues are eliminated from a small area on the mid-dorsal side of the cell.

3. Grains of fresh hard boiled yolk of hens egg, when prepared as described (page 8), furnishes a good unit of measure of the food taken, and an easy means for determining the factors which come into play in the process of feeding in *Bursaria*.

4. The amount of food eaten and the rate at which it is eaten depends upon the physiological state of the cell (defined on page 10). This is shown to be true for fresh and for fat-free yolk, and also for indigestible substances such as aluminium, Sudan III, chinese ink, etc.

5. Change in the physiological state of the cell is indicated by the change in the total amount eaten and the rate of feeding, under the same conditions.

6. The rate of feeding is not affected in proportion to the concentration of the yolk suspension.

7. Mechanical stimulation decreases the rate of feeding or inhibits it, roughly in proportion to the degree of stimulation.

8. Rise in temperature increases the rate of feeding on yolk.

9. Continuous action of white light of high intensity had no detectable effect upon feeding on yolk.

10. Feeding may continue during stimulation by a direct electric current of sufficient intensity to control the direction of movement of the organism.

11. *Bursaria* can discriminate between and select non-toxic grains of yolk from among toxic ones. Whether or not *Bursaria* will eat yolk grains that have adsorbed a soluble substance depends upon (a) the steepness of the effective concentration gradient of the dye, between the grain and the non-toxic medium; and this in turn depends upon the amount of dye adsorbed which is subject to a reversible adsorption; (b) the specific chemical properties ('taste?') of the substance adsorbed.

12. There are strong reasons for believing that different parts of the cell are affected unequally by certain toxic substances, and that these may have a specific action upon the selection mechanism, causing a more definite rejecting reaction.

13. Yolk which has adsorbed a substance which is insoluble in water (Sudan III) is eaten as readily as fresh unstained yolk.

14. *Bursaria* has the power of selective extrusion among vacuoles each containing different substances eaten at the same time; vacuoles containing indigestible substances are soon extruded, while those containing food are retained. If fat-free yolk is present in the same vacuole along with the indigestible substance, then the latter is retained until digestion of the enclosed yolk has run its usual course.

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THE ODD CHROMOSOME IN THE SPERMATOGENESIS OF THE DOMESTIC CHICKEN¹

ALICE M. BORING AND RAYMOND PEARL

SIX PLATES

The spermatogenesis of several birds has been described by Guyer: the pigeon ('00), the domestic guinea ('09 a), and the domestic chicken ('09 b). The chickens used were of the Black Langshan breed. Guyer reports for this form an X-chromosome, which goes into one half of the secondary spermatocytes, thus giving rise to two kinds of spermatozoa, half with X and half without. If this X-chromosome is assumed to be a sex chromosome, as in so many forms, the male bird, according to Guyer's account, must be heterozygous in regard to sex.

The chicken is one of the few animals on which there have been both breeding experiments and cytological work bearing upon this point. Breeding experiments with the domestic fowl have furnished a great mass of the clearest kind of evidence regarding the inheritance of sex. On account of the importance of the point at issue, and in order that the cumulative weight of the body of independent experimental evidence may be more readily appreciated, it will be well to review briefly the literature regarding sex-linked inheritance in poultry and in other birds. We may begin this review with the case of the inheritance of the barred color pattern of the Barred Plymouth Rock which has been thoroughly studied by a number of workers. Following the papers of Spillman ('09 a and b) suggesting, on the basis of observations of crosses of Black Langshans and Barred Plymouth Rocks, that the female fowl was heterozygous, and the

¹ Papers from the Biological Laboratory of the Maine Agricultural Experiment Station, No. 46.

male homozygous, for sex and the barred pattern factor, this was proved experimentally to be the case by Goodale ('09) who made crosses involving the following breeds; White Plymouth Rocks (in which barring is cryptomeric), Rhode Island Reds, and Buff Plymouth Rocks. These results were confirmed and extended in a series of papers from this laboratory by Pearl and Surface ('10 a, b, c) in which Barred Rocks and Cornish Indian Games were the breeds crossed. ♦ Hadley ('10) reported the same results with the same two breeds from earlier records obtained by Cushman. Morgan and Goodale ('12) have reported in detail the results of crossing Barred Rocks with Black Langshans (the breed used by Guyer in his cytological study) and American Dominiques with Langshans. Their findings are in entire accord with those of other workers. Davenport ('06) has reported a cross between White Cochins and Tosa, in which the results are clearly to be interpreted in the same way. Recently Hadley ('13) has reported White Leghorn \times Black Hamburg crosses, which again show that the female is heterozygous and the male homozygous for sex and barring (here carried as a cryptomere by the White Leghorn). Finally, it may be said before leaving the question of the barred pattern, that in unpublished work from this laboratory it has been shown that crosses of Barred Plymouth Rocks with (a) Campines, (b) Golden Pencilled Hamburgs, (c) Black Hamburgs, (d) White Wyandottes, and (e) White Plymouth Rocks, give results in every essential accordant with those already published regarding the inheritance of this color pattern.

Turning to other characters of poultry, breeding experiments have demonstrated sex-linked inheritance of the same sort as is shown by the barred patterns, in a fairly wide range of cases. Bateson and Punnett ('11) in crosses of Brown Leghorns (and several other breeds) with Silkies demonstrate sex-linkage of an inhibiting factor influencing the mesodermal pigmentation of the Silky. Goodale ('10) shows that the Brown Leghorn color pattern is sex-linked (crosses of Brown Leghorns with White Plymouth Rocks and White Langshans). Hagedoorn ('09) shows the same essential facts regarding 'Bankiva' and 'Brown-red'

color patterns in crosses of game bantams. Davenport ('11 and '12) reports the same sort of results for color pattern inheritance in Dark Brahma \times Brown Leghorn crosses. Sturtevant ('11 and '12) in crosses of Brown Leghorn and Columbian Wyandottes finds the female heterozygous for sex and certain sex-linked patterns and color factors. One of the present writers (Pearl '12) has shown that the factor on which high fecundity depends is sex-linked in the same manner as the barred color pattern (Barred Plymouth Rock and Cornish Indian Game breeds).

All of these cases, which cover a wide range of the different known types of domesticated Gallus, and include a considerable number of different hereditary characters, agree in showing that the female fowl is heterozygous for sex and sex-linked factors, while the male is homozygous. But Guyer's (loc. cit) interpretation of his cytological observations is flatly opposed to this body of clear-cut and definite evidence, based literally on thousands of cases and undergoing continued confirmation in several laboratories in the course of routine breeding operations primarily carried on for other purposes. As has been seen a number of independent investigations have checked, repeated and extended the experimental work with fowls, always with accordant and mutually confirmatory results.

Nor is this all. There is a considerable body of definite experimental evidence indicating that in other birds than Gallus the female is the heterozygous and the male the homozygous form. Here we have the investigations of Durham and Marryat ('08) with canaries, those of Staples-Brown ('12), Cole ('12) and Strong² ('12 a and b) with pigeons, and those of Goodale ('11) with ducks.

² Strong's results are included here for the following reasons: (a) his experimental facts appear to be in no essential particular different from those of other students of sex-linked inheritance in the pigeon; (b) a simple and direct interpretation of his results, in line with other cases of sex-linked inheritance, has been given by Bridges ('13); (c) because we are entirely unable to see the logical force of Strong's own interpretation, which makes the male heterozygous. Specifically it seems to violate that fundamental logical principle, which, as most adequately stated by Sir William Hamilton, runs: "neither more, nor more onerous, causes are to be assumed, than are necessary to account for the phenomena."

The history of the present investigation is as follows: In 1907-08 there was begun in this laboratory by Dr. F. M. Surface and one of the present writers (R. P.) a preliminary study of the spermatogenesis of the domestic fowl. The inherent difficulties of the material were at once apparent. It seemed clear that with any sort of technique then available it would be literally impossible to make out a definite and connected account of the spermatogenesis, in which the objective evidence on essential points would be unequivocal and indubitable. Later the other of the present writers (A. M. B.) took up the work. A great deal of time was spent in the development and experimental extension of technique in an attempt to overcome the inherent difficulty of the material. In 1910-11 the late Dr. N. M. Stevens was interested in the matter, and from then on till the time of her death in 1912 she devoted a very considerable amount of work to this problem, partly with material (both living and preserved) supplied from this laboratory and partly with cockerels of other breeds. After her death her notes, drawings and preparations were turned over to us. As will presently appear, Miss Stevens was totally unable to confirm Guyer's account in regard to any essential point. At the Cleveland meeting (1913) of the American Society of Zoölogists, Professor Guyer announced a demonstration of his chicken preparations showing the X-element. To one of us was given a very full account of the exact technique he had used. It may be said at once that every objective fact described by Guyer in his paper regarding the so-called X-element³ was clearly and successfully demonstrated. There can be no question regarding the existence of bodies which Guyer calls X-chromosomes. It is the chief object of this paper, however, to show that there is at present no valid evidence that any element which may justly be interpreted as an X-chromosome exists in the chicken.

³ No preparations were at hand for demonstrating the chromosome counts in the different stages of spermatogenesis, so this phase of the matter was not gone into.

MATERIAL AND METHODS

The material used in this investigation came from twelve pure Barred Plymouth Rock and crossbred males, raised on the poultry plant of the Maine Agricultural Experiment Station. The birds varied in age from five months to two years. Those younger than five months had no dividing germ cells. Beyond that time age seemed to have nothing to do with the number of dividing cells present, or the clearness of the mitotic figures. The testes of birds killed in the active breeding period, January to April, may perhaps contain more dividing cells, but no statistics were taken on this point, and those killed in October, November and December contained many such.

Three general methods of technique have been used in the preparations: stained sections, smears, and aceto-carmin mounts. The latter two give much better results than the first. The difficulties with much vertebrate germ cell material, as compared with insects, have been many times described, and the bird material is no exception. The chromosomes act as though they were of a sticky nature, and do not separate far apart even in prophase. Smears and aceto-carmin preparations both give a possibility of flattening out the cells, and for this reason give greater separation of chromosomes than any of the sections, no matter in what they are fixed. The material for stained sections was fixed mostly in Gilson's, Flemming's or Hermann's solutions. A testis of one bird was found in the laboratory fixed in Zenker-formalin; this was sectioned, but showed rather poor fixation. All possible schemes were tried to keep the cells in as normal condition as possible. The effort was made to transfer small pieces of the testes to the Gilson or Flemming solutions while the testis was still at the body temperature of the bird. For one bird, the Flemming and the Hermann solutions were heated to 38°C. when the pieces of testis were added in order to keep them at the normal temperature of the body of the bird until fixed. For two other birds, the whole testis hot from the bird's body, was immersed immediately in Flemming at 38°C., and cut into thin slices while in the hot solution so as to avoid any change

of temperature. But none of these precautions gained any better separation of the chromosomes. The Gilson's solution gave clearer fixation than either cold or hot Flemming or Hermann.

Very small pieces of the testes of some of these same birds were put into aceto-carmin and allowed to stay indefinitely. When examined later and spread one layer of cells thick under a coverglass, the chromosomes were sharper and clearer than in any of the sections of the material so carefully fixed. If 45 per cent acetic acid, saturated with carmin, gives good results, it seemed possible that other closely related organic acids or other concentrations of acetic acid might give even better chromosomal pictures. Consequently 45 per cent formic acid, 45 per cent butyric acid and 40 per cent chloracetic acid were tried. The butyric acid is too heavy and does not penetrate the cells at all. The formic acid fixed the cells well, but did not carry the stain in as well as the acetic. Of the different percentage solutions of the acetic acid tried, the 5 per cent did not either fix or stain well, the 100 per cent gave good sharp fixation, but would not dissolve enough carmin to stain properly. So nothing was found to improve on the 45 per cent aceto-carmin. Figures 86-91 are some of the aceto-carmin preparations.

Finally smears were made exactly as Guyer made his for the Langshan cocks. The testes were taken from an old bird in its second year. The smears were fixed in Bouin's solution and stained in iron hematoxylin. The black and white of the iron hematoxylin preparations is especially favorable for both drawing and photographing, and the smears are much better than the sections stained in iron hematoxylin. So figures 1 to 85 are all made from smear preparations.

OBSERVATIONS

This paper does not attempt to work out a continuous detailed history of spermatogenesis in the Barred Plymouth Rock. A glance at the photographs and drawings on plates 1 to 6 will show that such a history would require a large amount of imagination. These plates show, however, that in some of the dividing cells, a chromatin mass appears separate from the rest

of the chromatin on the spindle, and that this, if considered by itself apart from all other elements, resembles an *X*-chromosome. For instance figures 11 a and 51, 22 a and 73, show equatorial plates with a separate chromosome at one side; figures 14 and 61, 23 b and 82 show metaphase spindles with a chromosome toward one end of the spindle, apparently passing to one end without dividing. Figures 34, 65 and 83 show anaphases with projecting chromatin arms at only one pole. These three appearances are usually taken as signs that an *X*-chromosome is present. The more conclusive and unequivocal evidence based on the number of chromosomes in the spermatogonia, primary, and secondary spermatocytes respectively is not here available, because it is impossible to count chromosomes accurately in this material.

However, to follow out the only kind of evidence available, it is clear that half the above sample figures represent primary spermatocytes, and half secondary. Now the typical *X*-chromosome fails to divide in only one spermatocyte division: either the first, as in *Coleoptera* (Stevens '06) and *Hemiptera homoptera* (Boring '07) or in the second, as in some *Hemiptera heteroptera* (Wilson '05), and *Orthoptera* (McClung '00). It behaves like any other chromosome in the equatorial maturation division, whichever that may be. But the apparent *X*-chromosome in the Barred Plymouth Rock can be seen passing undivided to one pole in both primary (fig. 65) and secondary (fig. 83) spermatocytes. This would result in one-quarter of the spermatozoa with *X* and three-quarters without it. Following this further; if the chicken were like the majority of forms with sex chromosomes so far studied, and had one less chromosome in the male than in the female, there would be on the basis of the above observations a sex ratio among chickens of one female to three males. Davenport's ('06) work on poultry, as well as very extensive unpublished statistics on the point in the records of this laboratory, show that such a ratio does not exist in this form.

Another possibility is that the *X*-chromosome may divide in either the first or second spermatocyte division. Edwards describes a case of this in *Ascaris* ('10), but this is a rare occurrence.

That this is not the explanation in the Barred Plymouth Rock will be shown by the statistical tables given later in this paper (pp. 61-62).

It is interesting to notice that it is easy to pick out from either the photomicrographs or the drawings a series of pictures which, taken by themselves, would show an *X*-chromosome to be present and to find it dividing in the first maturation division. Figures 26 a, 30, 34, 36 c, 35 a, 35 b are such a series of photographs, and figures 45, 62, 66, 70, 78, 84 such a one of drawings. Further, figures 3 a, 17 a, 20, 22 a, 23 b, and the series 39, 54, 63, 77, 82, 83, taken by themselves, show just as conclusively that an *X*-chromosome is present and fails to divide in the second spermatocyte division. Still further, a series of figures made by a combination of the figures of the previous two series, for example, figures 3 a, 17 a, 20, 36 c, 35 a, 35 b, or 39, 54, 63, 70, 78, 84 shows that there is no *X*-chromosome present at all.

The above reductio ad absurdum, proving three conflicting hypotheses out of the same material, is the crux of the argument of this paper. In difficult cytological material, such as this, one can prove anything one wants, by picking out certain cells and ignoring all others. The only way to find out the real facts is to study a large number of cells and find out what happens in the majority. So this investigation comes to a focus around the question: Is this so-called *X*-chromosome in the Barred Plymouth Rock present in enough cells to be justly regarded as the homologue of the *X*-chromosome in insect spermatogenesis?

The following statistics were worked out to answer this question. Slides were systematically examined by means of the mechanical stage, and every clear dividing cell within a certain area was recorded under one of the twelve headings in table 1. Several nuclei are often grouped in one individual mass of cytoplasm, as described by Guyer for the Langshan birds ('09). It is easy to distinguish the second spermatocytes from the first by the amount of chromatin and the size of the spindle. The criteria used for the presence of *X* were those used by Guyer for the Langshan material, either a separate mass of chromatin as in figures 10 and 14, or a very conspicuous arm protruding

from the mass, as in figures 1 and 13. On this basis, 1003 cells were grouped into six classes with *X* and six classes without *X*. The six classes are (1) first spermatocyte, equatorial plate; (2) first spermatocyte, metaphase spindle; (3) first spermatocyte, anaphase; (4) second spermatocyte, equatorial plate; (5) second spermatocyte, metaphase spindle; and (6) second spermatocyte, anaphase. The results are given in table 1. These figures plainly show that the so-called *X*-chromosome is an exception rather than a rule. Eighty-one cells with *X* out of 1003, that is 8.07 per cent, is obviously too few to justify a generalization as to the specific character of the element, even with all the possible chances of its escaping notice. This table also gives numerical expression to the difficulty before mentioned, that *X* appears in

TABLE 1

CELLS OF TESTIS OF BARRED PLYMOUTH ROCK	I SPC. EQ. PL.	I SPC. META.	I SPC. ANA.	II SPC. EQ. PL.	II SPC. META.	II SPC. ANA.	TOTAL I SPC.	TOTAL II SPC.	TOTAL
No. of cells .	267	245	63	120	248	60	575	428	1003
No. of cells with <i>X</i> ..	40	23	5	6	5	2	68	13	81
No. of cells without <i>X</i>	227	222	58	114	243	58	507	415	922
Per cent with <i>X</i> .	14.97	9.38	7.93	5.00	2.01	3.33	11.82	3.06	8.07

both first and second spermatocyte divisions. If this were a form where *X* passed undivided to one pole in the first spermatocyte division, 11.82 per cent cells showing it in the first spermatocyte would be too few, and 3.06 per cent in the second spermatocyte would be too many. If on the other hand this were a case where the odd chromosome failed to divide in the second spermatocyte division, 11.82 per cent cells showing it in the first spermatocyte division would be too many, and 3.06 per cent too few. So the actual facts will fit neither case. And we see now why the situation found by Edwards in *Ascaris* where the *X*-chromosome divides sometimes in the first spermatocyte division and sometimes in the second, will not answer here: 8.07 per cent is still not enough cells with *X*, even allowing for this possibility of variation in behavior.

In the last paragraph it was assumed that *X* would not escape notice in many of the cells of a form where present, that is, that the element would be visible if present, in approximately 50 per cent of all dividing spermatocyte cells, 100 per cent of first and 0.00 per cent of second spermatocyte cells, or vice versa, 100 per cent of second and 0.00 per cent of first. But any one who has worked much in the cytology of germ cells knows that there are good reasons why *X* sometimes escapes notice when present. It does not by any means always take up an eccentric position in the equatorial plate; it does not always show itself in the side view of the metaphase spindle by projecting at one side or by appearing visibly univalent instead of bivalent; neither does it

TABLE 2

CELLS OF TESTIS OF PHILAENUS SPUMARIUS	I SPC.	I SPC.	I SPC.	II SPC.	II SPC.	II SPC.	TOTAL	TOTAL	TOTAL
	EQ. PL.	META.	ANA.	EQ. PL.	META.	ANA.	I SPC.	II SPC.	
No. of cells	22	128	423	34	69	324	573	427	1000
No. of cells with <i>X</i> ..	14	111	369	0	0	0	494	0	494
No. of cells without <i>X</i>	8	17	51	34	69	324	79	427	506
Per cent with <i>X</i>	63.63	88.43	87.23	0	0	0	86.21	0.00	49.4

always lag behind the dividing chromosomes during the anaphase of the mitosis. In order, then, that the conclusions of the previous paragraph might be based on facts and not on an assumption, a similar statistical study was made of 1000 dividing cells of a bug, *Philaenus spumarius*, the spermatogenesis of which has been previously worked out and found to exhibit a typical unpaired sex chromosome (Boring '13). The cells were picked out in the same way with the mechanical stage, systematically at random, all the clear ones in a certain area being catalogued. The results are seen in table 2, which shows that the previous assumptions were indeed very close to the facts. Out of 1000 cells, 49.4 per cent show *X*, none of the second spermatocytes show it as a chromosome behaving differently from the others, while in 86.21 per cent of the first spermatocytes, it can be differentiated. In the equatorial plate, there were only 8 out of 22

cases, where the *X*-chromosome was not eccentric. On the metaphase spindle, there were 17 out of 128 cells, where the bivalent character of the other chromosomes was not striking enough to make the difference visible. Among the anaphases, only 54 out of 423 cells failed to show a lagging chromosome at one pole; in these it had probably already reached its destined pole and lay in the same plane with the others. A comparison of these figures with those for the Barred Plymouth Rock are certainly convincing: the Barred Plymouth Rocks do not show enough cells with an apparent *X*-chromosome to make it even mildly probable that this is a sex chromosome like the *X*-chromosome of insects. In the Barred Plymouth Rock there are 11.82 per cent primary spermatocyte cells with a possible *X*-chromosome, as contrasted with 86.21 per cent in the insect. In the secondary spermatocytes, the Barred Rock has 3.06 per cent cells with an apparent *X*, while the insect has 0 per cent.

We have seen then that the small proportion of cells with a possible *X*-chromosome casts some doubt on its interpretation as such. We will examine next some of the individual cells where this separate mass of chromatin appears, and see whether it is always consistent in shape and size, that is, whether it can be regarded as a definite individual chromosome of any kind whatsoever.

The figures, both photomicrographs and drawings, plainly show that the shape of the separate mass of chromatin varies. It may be round, as in figures, 11 a, 12 b, 14, 16, 22 d, 27, 28 a, 28 c, 50, 77 and 82. It may be rod-shaped, as in figures 5, 8 b, 34, 49, 52, 53, 57, 65. It may be *U* or *V*-shaped, as in figures 9, 33, 46, 47, 48, 60, 61. It may be three-parted, as described by Guyer ('09) for the Langshan cock, and shown in the following figures of this paper: 10, 26 b, 43, 44, 74, 83. It may even be four-parted, as in figures 45, 62, 66 and 73. Besides these, there are many cells where rod-like arms project from the mass of chromatin, which are rated by Guyer as *X*-chromosomes (figs. 1, 13, 29, 38, 40, 55, 56, 81).

Even more striking than the variation in shape is the variation in size. This ranges all the way from a tiny speck (figs.

2 b, 2 c, 14, 49, 51) up to a piece almost half the volume of the whole chromatin mass (figs. 7 b, 8 b, 25 b, 25 c, 43, 75).

Beside the above-mentioned inconsistencies in shape and size, in some cells there is more than one separate piece of chromatin. Figures 10, 27, 48, 49, 53, 83 show two separate chromosomes, and figure 28 c shows three, while figure 75 may show four. These are cases where the two or three *X*-chromosomes are all distinctly separated from the chromatin plate. If one counts the projecting arms and pieces lying close to the large mass, yet apparently separate chromosomes, there are many more cases with more than one *X*-chromosome. Figure 1 has four arms; figure 28 a has one round *X*-chromosome and one arm; figure 46 has one *V*-shaped chromosome and one arm; figure 49 has one round odd chromosome, one rod-shaped and one arm; figure 52 has one separate rod and one rod-like arm; figure 61 has one *V*-shaped element and two arms. If it is legitimate to call an arm an *X*-chromosome when there is no separate chromosome to call *X*, then it ought to be equally legitimate to count an arm as an *X*-chromosome, when a separate chromosome is present, and to conclude that there is more than one *X*-chromosome in such cases.

A study of the anaphases shows that in this Barred Plymouth Rock material, a lagging condition, as indicated by a projecting arm cannot safely be regarded as indicating a real difference in behavior from other chromosomes, for there are in actual fact as many cases with corresponding lagging arms at both poles of the anaphase spindle as with one at only one end. Figures 18 b, 19, 21, 67, 68, 69 are spindles where this is the case. This evidently means merely that these Barred Plymouth Rock chromosomes do not always separate as evenly and as synchronously as those in insect material.

We have seen that the so-called *X*-chromosome of the Barred Plymouth Rock male germ cells varies in shape, size and number. If it is such a variable element, it is evident that many of the figures used in this paper are capable of varying interpretations. Let us run over the figures to see what possible interpretations we find for some of them. Figure 1 is a primary spermato-

cyte equatorial plate. It has four projecting arms. Perhaps these are four *X*-chromosomes. But two arms are longer than the others. Perhaps only the two longer arms are *X*-chromosomes. The two long arms are rod-shaped, while the two short ones are rounded. If there is only one *X*-chromosome and that is round, it must be one of the short arms; if it is rod-shaped, it must be one of the longer. If there are two *X*-chromosomes, one might be rod-shaped and one round, or both might be round, or both might be rod-shaped. Or it might much more probably be that there is no *X*-chromosome at all, and all four arms are only ordinary bivalent chromosomes, which happen to project a little from the plate of chromosomes.

Figure 4 is a primary spermatocyte equatorial plate and may have a round *X* to the left and a rod-shaped one to the right, or either one of them may be *X*, while the other is a bivalent chromosome. Or again, there is nothing about either which stamps it even probably as an *X*-element, so there may be no *X* at all.

Figure 12 b is a first spermatocyte equatorial plate and may have either one or two *X*-chromosomes, according to whether one counts the round body which touches the mass of chromosomes as one or not. It does not happen to be quite as far away as the entirely separate one, but there is no other indication of its being different in character.

Figure 13 is a side view of a primary spermatocyte metaphase spindle, the arm to the left, pointing toward the upper pole may represent an unpaired chromosome, but on the other hand, there is no surety that this is univalent, while the rest of the mass is composed of bivalent chromosomes.

Figure 33 is a first spermatocyte metaphase spindle. The body to the right may be a *V*-shaped *X*-chromosome, or it may be a three-parted *X*-chromosome, or it may be an ordinary dumb-bell-shaped bivalent chromosome in the process of dividing.

Figure 18 b is a first spermatocyte anaphase. Any one of the three arms to the left might be a lagging *X*-chromosome, but the general scattered condition of the chromatin would make it just as reasonable to interpret all three as ordinary chromosomes. The middle one at least has a mate at the right pole. When

the microscope is focused on the right pole instead of the left, arms appear which might be considered the mates of the other two arms of the left pole.

Figure 66 is also a first spermatocyte anaphase. The three or four-parted body toward the upper pole might be an X-chromosome, but the chromosomes are so loosely grouped here, and the separate body is so close to the pole that such an interpretation has no particular probability in its favor.

Figure 22 a is a second spermatocyte equatorial plate. The body to the left may be one rod-shaped X-chromosome, or two round ones, or not an X-chromosome at all.

Figure 81 is a second spermatocyte metaphase spindle. The part projecting toward the lower pole might be a univalent X-chromosome, or one part of a large bivalent dividing.

Figure 83 is a second spermatocyte anaphase. The three-parted body at the lower pole may be an X-chromosome, or it may be the mate to the projecting mass at the upper pole. The small round chromosome at the lower pole may be a second X-chromosome, or it may be one of the ordinary chromosomes with its mate fused in the mass at the upper pole.

Similar varying interpretations might be given for most of the figures. There would then appear to be abundant justification for the statement that there is no valid evidence of the existence of an X-chromosome in the Barred Rock male, on the following grounds:

- a. The small percentage of cells containing any element which, on the most liberal interpretation, could be regarded even as a possible X-chromosome.
- b. The inconstancy of the shape, size and number of these possible X-chromosomes.
- c. The fact that many (not to say most) of them would never be considered to be sufficiently proved as X-chromosomes if this were insect material.

The 11.82 per cent of first spermatocytes and the 3.06 per cent of second spermatocytes classified in the table as 'with X,' however, demand further attention. The projecting arms and those lying in close juxtaposition to the equatorial plate have

had an explanation offered for them in the above paragraphs, but there are some cells such as figures 10, 14, 28 c, 53 and 61 where there is a distinctly separated piece of chromatin. It seems, however, a reasonable explanation that these are one, two, or several of the ordinary chromosomes that happen to be too far from the others to fuse into the general mass. If this material were such that one could count the number of chromosomes in the first and second spermatocytes, and see tetrads formed for division, one could tell absolutely whether the pieces were extra chromosomes or not. But the chromosomes act almost as though they were sticky on the surface, and those that happen to touch each other stick close together and stain as though fused. Comparing figure 53 with 38 it is clear that the right hand piece is no further from the center of the mass in the first than in the second case, only it is one of the smaller chromosomes and therefore did not touch the mass and stained separately. If one compares figures 52 and 40, the same thing is seen: the rod is a little shorter and does not quite touch the mass in 52, while it just touches it in 40.

A study of all the photographs of the primary spermatocyte equatorial plates as shown on plate 1 and all the drawings of the same stage, as shown on plate 4, in the order in which they are arranged on the page will show a gradual transition from the large mass with projecting arms, through the smaller mass with separate chromosomes nearby or even touching, to the small mass with chromosomes quite far away. A similar study of the series of photographs and drawings of the side views of the metaphase spindles of the first spermatocytes, as shown on plates 2 and 5, indicates the same sort of transition.

The difference in size of these chromosomes left outside of the general mass in different cells, would indicate a regular and constant size difference among the chromosomes. Some of the clearest aceto-carmin preparations give additional evidence for this (figs. 87, 89, 90, 91). In these preparations it was more clearly possible to see all the separate chromosomes than in preparations by any other technique. But even here counting proved illusory. Figure 86 shows six chromosomes, figure 87 seven,

figure 88 eight, and figures 89, 90 and 91 nine. Figures 50 and 85 are the only two cells from smear preparations, where counting was at all possible, and here it seems rash. Both of these cells seem to show nine chromosomes. In the notes and rough sketches left by Dr. N. M. Stevens on the Barred Plymouth Rock material she has recorded more cells where she could count nine than any other number, but she had counts recorded all the way from 5 to 22.

Another note of Dr. Stevens indicated that she did not find any sign of the double reduction recorded by Guyer for pigeons, chickens and guineas. Figures 70 to 75 look as though the number of chromosomes present in the secondary spermatocytes must be more than half of nine, and figure 90, an aceto-carmine preparation of a secondary spermatocyte, shows nine clearly as the second spermatocyte number. However, these facts as to number are too meager to be offered as anything more than isolated observations. The result of this investigation is to show that there is no good evidence for a sex chromosome in the germ cells of the Barred Plymouth Rock males and that therefore there is no cytological evidence to conflict with the experimental evidence that the female fowl is heterozygous and the male homozygous for sex.

SUMMARY

There is no good observational or statistical evidence of the existence of a sex chromosome in males of domestic chickens of the Barred Plymouth Rock breed. In 11.82 per cent of first spermatocytes and 3.06 per cent of second spermatocytes, there is a piece of chromatin similar to that called an X-chromosome by Guyer in Langshan males. This is not to be regarded as an X-chromosome in Barred Plymouth Rock males, because:

1. It is present in spermatocytes of both orders.
2. A statistical study of Barred Plymouth Rock cells in comparison with those of the hemipteran *Philaenus spumarius* shows that it is present in too few I spermatocytes and in too many II spermatocytes, or vice versa.

3. It is still present in too few cells, if it should be interpreted as one of those rare cases where the odd chromosome divides in either the I or II spermatocyte division.

4. It varies in shape.

5. It varies in size.

6. It varies in number.

7. In no single cell is it of such a shape or size, or in such a position, that it could not readily be interpreted as anything else than an X-chromosome.

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EXPLANATION OF FIGURES

The photomicrographs of plates 1 and 2 were made with a Leitz $\frac{1}{2}$ oil immersion and 6 ocular, and enlarged in the taking so that they give a magnification of 1150. Those of plate 3 were made with a Zeiss 2 mm. objective and 6 ocular, giving a magnification of 750. The drawings of plates 4 to 6 were made with a camera lucida, at the level of the table, and with a $\frac{1}{2}$ oil immersion, and 12 compensating ocular, no reduction. Figures 1 to 85 inclusive, both photographs and drawings are from smear preparations fixed in Bouin and stained in iron hematoxylin. Figures 86 to 91 are of aceto-carmin preparations. The photographs were made by Mr. Royden L. Hammond.

PLATE 1

EXPLANATION OF FIGURES

First spermatocyte division stages

- 1 Equatorial plate with four projecting arms.
- 2 a Equatorial plate with one projecting arm.
- 2 b and 2 c Metaphase spindles, each with a very small separate chromosome.
- 3 a Equatorial plate, chromosomes closely massed together
- 3 b Metaphase spindle with no X-chromosome
- 3 c Anaphase with projecting arm at the left pole
- 4 Equatorial plate with a round chromosome to the left and a rod to the right.
- 5 Equatorial plate with a rod to the right
- 6 a Equatorial plate, chromosomes much scattered
- 6 b Anaphase with no possible X-chromosome.
- 7 a Equatorial plate, chromosome is fused.
- 7 b Equatorial plate, chromosomes in two nearly equal masses.
- 8 a Metaphase spindle with no possible X-chromosome.
- 8 b Equatorial plate, large separate rod, one-third the size of whole chromosome mass.
- 9 Equatorial plate, V-shaped chromosome to right
- 10 Equatorial plate, two separate chromosomes, one round to left, and one three-parted to right.
- 11 a Equatorial plate, one round chromosome to the right
- 11 b Equatorial plate, separate round mass, one-third the size of the whole chromosome mass.
- 12 a Equatorial plate, no possible X-chromosome.
- 12 b Equatorial plate, two separate round chromosomes to the right.

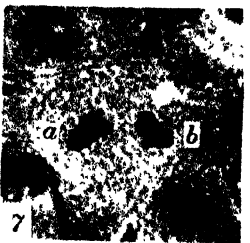
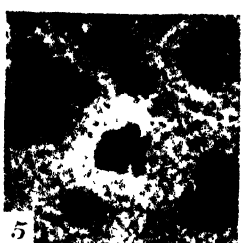


PLATE 2

EXPLANATION OF FIGURES

13 to 18 a First spermatocyte metaphase spindles

- 13 Extra arm to left toward upper pole.
- 14 Small round separate chromosome toward lower pole.
- 15 and 16 Round chromosome projecting from the mass toward the lower pole.
- 17 a and b No possible X-chromosome present.
- 18 a No possible X-chromosome present.

18 b to 21 First spermatocyte anaphases

- 18 b Left pole of spindle more clearly in focus than right; at least one of three projecting arms at the left has a corresponding arm at the right.
- 19 Two projecting arms at upper pole, and one at the lower.
- 20 No possible X-chromosome.
- 21 Projecting arm at right of both upper and lower poles.

22 and 23 Second spermatocytes

- 22 a Equatorial plate, rod to left.
- 22 b Metaphase spindle, small round projection toward lower pole.
- 22 c Equatorial plate, no possible X-chromosome.
- 22 d Equatorial plate, separate round chromosome to left.
- 23 a Anaphase, no possible X-chromosome.
- 23 b Metaphase spindle, small round projection toward lower pole.

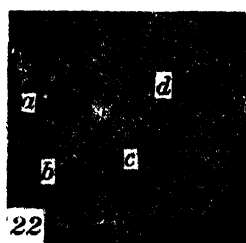
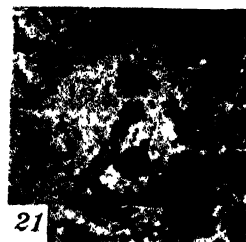
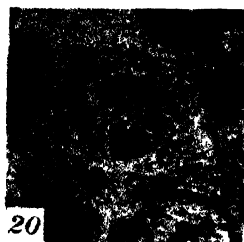
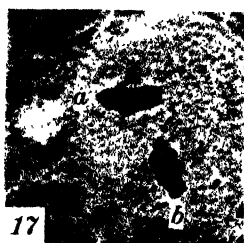
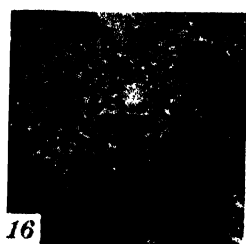
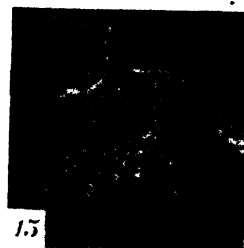


PLATE 3

EXPLANATION OF FIGURES

24 to 34 First spermatocyte divisions

- 24 a Metaphase spindle, projecting arm toward left pole.
- 24 b Equatorial plate, projecting arms.
- 25 a Equatorial plate, no possible X-chromosome
- 25 b and c Equatorial plates, mass of chromosomes in two pieces.
- 26 a Equatorial plate, round chromosome to right.
- 26 b Metaphase spindle, three-parted chromosome toward upper pole.
- 27 Equatorial plate, two separate round chromosomes.
- 28 a Metaphase spindle with round chromosome to the right, and an arm to the left.
- 28 b Metaphase spindle, one small round chromosome to left.
- 28 c Equatorial plate, three separate round chromosomes.
- 29 Metaphase spindle, corresponding projecting arms to the right toward each pole.
- 30 and 31 Metaphase spindle, one round chromosome toward lower pole.
- 32 Metaphase spindle, projecting arm toward upper pole, and round chromosome toward lower.
- 33 Metaphase spindle, V-shaped chromosome to right.
- 34 Anaphase, projecting rod-like arm only at lower pole.

35 to 37 Second spermatocyte divisions

- 35 a Metaphase spindle, no possible X-chromosome.
- 35 b Anaphase, no possible X-chromosome.
- 36 a and d Equatorial plates, out of focus.
- 36 c and e Equatorial plates, no possible X-chromosome.
- 36 b Anaphase, no possible X-chromosome.
- 37 a and c Equatorial plates, out of focus.
- 37 b Metaphase spindle, no possible X-chromosome.
- 37 d Equatorial plate, round chromosome toward upper side.



PLATE 4

EXPLANATION OF FIGURES

38 to 53 First spermatocyte equatorial plates

- 38 Chromosomes massed with projecting arms.
- 39 Chromosomes loosely massed with space in center.
- 40 Chromosomes scattered, but still all connected.
- 41 Chromosomes massed, with a V-shaped body partially separated to the right.
- 42 Chromosomes massed, with a three-parted body partially separated to the right.
- 43 Three-parted separate body, one-third the size of the whole chromosome.
- 44 Three-parted separate body, small in proportion to size of whole chromosome plate.
- 45 Four-parted separate chromatin body to the right
- 46 V-shaped separate chromatin body to right.
- 47 V-shaped separate chromatin body at lower side.
- 48 Two separate chromosomes, one round and one V-shaped.
- 49 Two separate chromosomes, one small round one and one rod-shaped.
- 50 One round chromosome somewhat separated from the rest. The number of chromosomes here might be counted as nine.
- 51 One small round separate chromosome to the right.
- 52 Two rod shaped chromosomes, one separate and one attached.
- 53 Similar to 52, but rods shorter.



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PLATE 5

EXPLANATION OF FIGURES

54 to 62 First spermatocyte metaphase spindles

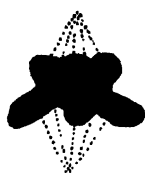
- 54 No possible X present.
- 55 and 56 Two projecting arms.
- 57 One projecting rod toward lower pole.
- 58 and 59 Projecting round chromosome toward lower pole.
- 60 V-shaped chromosome toward lower pole.
- 61 V-shaped chromosome toward lower pole and two projecting arms toward upper pole.
- 62 Four-parted body toward lower pole.

63 to 69 First spermatocyte anaphases

- 63 No possible X-chromosome present
- 64 Projecting arm at upper pole.
- 65 Projecting rod-like arm at lower pole.
- 66 Separate four-parted body at upper pole.
- 67 to 69 Corresponding projecting arms at both poles.



54



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56



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58



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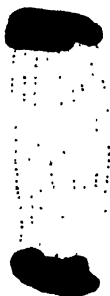
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PLATE 6

EXPLANATION OF FIGURES

70 to 84 Second spermatocyte divisions

70 to 72 Equatorial plates, no separated chromosome. Apparently more than one-half as many chromosomes as in I spermatocyte, although counting is impossible.

73 to 77 Equatorial plates, with separated chromosomes, varying in number and shape.

78 and 79 Metaphase spindles, no possible X-chromosome.

80 and 81 Metaphase spindles, with projecting arms toward lower pole.

82 Metaphase spindle, one round separate chromosome toward lower pole.

83 Anaphase, two separate chromosomes toward lower plate, one round and one three-parted.

84 Anaphase, no X-chromosome present.

85 First spermatocyte equatorial plate. One of the few cells in the smear preparations where it is possible to try to count chromosomes, the number might be nine.

86 to 91 Aceto-carminc preparations

86 First spermatocyte equatorial plate, where there are apparently 6 chromosomes.

87 First spermatocyte equatorial plate, where there are apparently 7 chromosomes.

88 First spermatocyte equatorial plate, where there are apparently 8 chromosomes.

89 First spermatocyte equatorial plate, where there are apparently 9 chromosomes.

90 Second spermatocyte equatorial plate, where there are apparently 9 chromosomes.

91 First spermatocyte anaphase, where there are apparently 9 chromosomes at each pole.

SPERMATOGENESIS OF THE CHICKEN

ALICE M. BORING AND RAYMOND PEARL

PLATE 6



70



71



72



73



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89



90

THE EFFECT OF RADIUM RADIATIONS ON THE FÉRTILIZATION OF NEREIS

CHARLES PACKARD

THREE PLATES

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INTRODUCTION

The radiations of radium have been used as a stimulus to protoplasmic activities from the time they were first discovered. The early experiments were made with little knowledge of the nature of the stimulus and with none whatever as to its probable effect on living matter. It was found that the rays affected the tissues in very different ways, acting as a stimulus to growth in some tissues, and as an inhibitor in others. Thus, seedlings have, in some instances, been accelerated in growth, and the action of certain enzymes (pepsin, diastase, etc.) favored by exposure to the rays. On the other hand, an exposure to radium produces a marked retardation in the growth of certain tissues and a characteristic degeneration in some of the cell constituents. The latter effects

are found chiefly in the rapidly growing structures, such as embryonic or regenerating tissue. The nature of the injury has been studied particularly in the former type, and the generalizations made on the effect of radium radiations have been based chiefly on such investigations. A study of the cytological details of the changes thus brought about has been the point at issue in the most recent studies on this subject and is the object of the present investigation.

THE NATURE OF THE RADIUM RADIATIONS

A discussion of the nature of the radium radiations need not be entered into fully in this paper. It may be mentioned simply that the three types of rays apparently produce rather different effects. The alpha rays, which form the greatest part of the energy given off, are wholly shut out by the glass or mica screens which are usually arranged for the protection of the salt. As the apparatus used in the following experiments and in the investigations of all those who used living tissues as an object for study effectually screened the alpha rays, they may be disregarded in this discussion. The beta and gamma rays are able to penetrate thin glass, but their activity is greatly diminished in the transit. Their effect on protoplasmic activities is, in general, an injurious one, although Congdon ('12) found some evidence of acceleration in the rate of development in the eggs of *Drosophila* due to the secondary gamma rays. In this case only the eggs which received a very weak stimulus were accelerated. The injurious effects found by practically all observers will be discussed in the next section.

HISTORICAL REVIEW

It has been found that different tissues show a great diversity in the nature of their response to the radiations of radium. Thus, the spermatogonia and the spermatocytes in the testis of the rat may be killed or greatly injured while the surrounding Sertoli cells and the connective tissue show no effects whatever. The same is true for ova and the follicle cells. Körnicke ('05) who exposed to radium the growing root tips of *Lilium* for periods of

one hour to three days, found that the most noticeable effect was on the nucleus of the cells, the protoplasm apparently undergoing no change. The chromatin was clumped together, and in some cases formed a homogeneous ball. He also found in the tetrad cells a great number of small nuclei, and many extra-nuclear nucleol'.

Guilleminot ('08) found similar changes. With intense radiation (200 mg. of the pure bromide) the nucleus of the pollen grain lost all power of division after the fertilization of the ovule. With weaker stimulus the resulting embryo was small and abnormal in various respects. If the stimulus is applied during fertilization the injurious effect appears early in development, and the plant is unable to repair the injury. If the adult tissue of the plant is exposed, very little effect can be noted.

Experiments on the effect of radiations on the germ cells and on the fertilized egg have been carried on by numerous observers. The first of these was Bohn ('03) who used the frog and sea-urchin as the basis of investigations. In general he found that the radiations produce a retarding and injurious effect on the early development of both of these forms. On exposing sea-urchin sperm he found that they very soon lost their motility and failed to fertilize the eggs, a fact not borne out by the experiments of G. Hertwig in the case of *Parechinus miliaris*, or by my own on *Arbacia punctulata*.

Perthes ('04) has described similar effects of radiation on the fertilized eggs of *Ascaris megalocephala*. Schaper ('04) and Levy ('06) have described the abnormal larvae arising from the radiated, fertilized frog's egg. The abnormalities consisted in the peculiar shape of the larvae, and the pathological condition of the nervous system. The vascular system also was much injured. Schaper believed that the abnormalities in the embryo were due in a large measure to the destructive action of radium radiations on the yolk, the lecithin being destroyed through the ionizing effect of the radiations. This theory, first proposed by Schwarz ('03), will be discussed later.

Bardeen ('11) has found that X rays (which are similar to the gamma rays of radium) produce a marked effect on both eggs and sperm cells of frogs so that they give rise to abnormalities. The

radiation of the egg produces a greater injurious effect than that of the sperm. In early cleavage susceptibility of the cells is increased, but in later stages he found, in common with many other observers, that the susceptibility is greatly diminished.

A more critical examination of the effect on the embryo of radiating the germ cells has been made by O. and G. Hertwig who also used the frog as a basis of investigation. In four series of experiments they exposed first the fertilized eggs (*A* series); the sperm (*B* series); the unfertilized egg (*C* series); and both eggs and sperm before fertilization (*D* series). In general they found that the exposure of the egg or sperm alone produced smaller abnormalities than the exposure of the fertilized egg. In the *A* series and in the *D* series the development was affected in proportion to the length of exposure and the intensity of the radiation. The same is true, up to a certain point, for the *B* and *C* series. But if the sperm or the egg is exposed for a long time the resulting embryo is fairly normal. In such cases, according to O. and G. Hertwig, development is parthenogenetic, since the sperm nucleus never becomes a part of the cleavage nucleus, but serves merely to initiate development. The same is true when the egg is radiated. The sperm nucleus and not the egg nucleus is active.

The embryos developing after the various types of treatment show characteristic abnormalities. Gastrulation may be very abnormal and the older embryos much bent, as described by Schaper ('04). The internal structures also show great changes. Most seriously affected is the nervous system in which the cells are abnormal and the nuclei broken into granules. The blood system also is injured. Physiological abnormalities are as marked as the morphological. Growth is greatly retarded, due to the slow rate of cell division. Though the muscles develop they are paralyzed for lack of an adequate nervous system. The nuclear disturbances in the affected cells are similar to those found by earlier investigators, but the plasma of the cells appears to be unchanged.

A further study of the exact nature of the injury to the nucleus has been made by P. Hertwig ('11) and G. Hertwig ('12). The

former found that the fertilized egg of *Ascaris megalocephala*, after exposure to radium, shows much the same type of abnormality that had been observed by others, namely, that the chromatin alone is affected, being broken up into numerous irregular granules. In division these granules are not exactly equally divided, but part go to one blastomere and part to the other. The astral system and the plasma are not affected at all. Miss Hertwig concludes that the effect of the radium radiations is a direct one on the chromatin, and not indirect, as Schwartz and Schaper claimed.

G. Hertwig ('12) has studied the effect of fertilizing the normal sea urchin egg with radiated sperm. The sperm remains active after twelve hours of intense radiation. But such sperm, even though motile and able to penetrate the egg, is incapable, in many instances, of fusing with the egg nucleus. Inside the egg it remains as a compact mass near the latter which divides, being provided with centrosomes derived from the sperm. In some instances it may become involved as a foreign body in the spindle of the dividing egg nucleus, in which case it causes abnormalities in the distribution and form of the egg chromosomes. In division it usually goes to one blastomere, and in later divisions may operate to produce very abnormal mitoses resulting in an abnormal larva. In other instances it may fuse with the egg nucleus but even then it behaves abnormally and is eliminated during the subsequent divisions. The main point is that the embryos arising from this treatment are parthenogenetic. Thus cytological study has confirmed his earlier hypothesis based on the experiments with the frog's egg

Since there is no visible evidence that the protoplasm of the sperm or fertilized egg has been injured, the Hertwigs conclude that only the chromatin is affected. This conclusion is supported by the fact that the harmful effect of the radiations is much greater in the fertilized egg, with twice as much chromatin, than in the normal egg fertilized by radiated sperm.

Two hypotheses have been advanced to explain the phenomena that have been described. The first, proposed by Schwarz ('04) was based on the fact that egg yolk is decomposed by exposure to the

radium radiations. Although the matter was not chemically determined, it seemed probable that the lecithin was broken up into cholin and tri-methyl-amine and other end products of lecithin decomposition. Lecithin has been found by many investigators in all cells, especially in egg yolk, spermatozoa, pollen cells, plant spores, growing buds, and in all rapidly growing tissue. If then, it is destroyed such cells must necessarily be unfavorably affected.

Against this hypothesis can be raised a number of objections. In the spermatozoön there can be very little lecithin, and any amount that is destroyed would be so small as to be nearly negligible. Yet Hertwig has stated that exposure of frog sperm for one minute is sufficient to produce some abnormality in the embryo. It is hard to imagine that this is due to the amount of lecithin decomposed in so brief a period. On the other hand, the egg contains a relatively enormous quantity, so that the effect should be correspondingly greater, yet such is not the case in the frog. The experiments of Bardeen show only a slightly greater injury when the egg is exposed. It is also found that the fertilized egg, when radiated, develops very abnormally even though the exposure is brief. These facts indicate that the lecithin hypothesis cannot explain all of the phenomena although the decomposition of the lecithin under radiation is an actual fact.

The second hypothesis, advanced by O. Hertwig is called by its propounder a 'Biological hypothesis' although fundamentally chemical in its nature. It is assumed that the radium radiations affect only the chromatin and that all the abnormalities that result from radiations arise in consequence to the injury to that substance. The chromatin, under the influence of the radiations produces a 'contagium vivo' which acts like a living ferment in that it increases at each cell division. Thus an originally small amount generated in the sperm will make itself felt after many cleavages, while a large amount generated in the fertilized egg will produce abnormalities almost at once. In cases in which the spermatozoa have been radiated for a long time it is assumed that the 'contagium vivo' has increased to such an extent that it has killed itself and at the same time affected the dividing power of

the chromatin. The destruction of the poison by prolonged radiation makes it possible for the egg to develop parthenogenetically. When the fertilized egg is radiated enough poison is generated to prevent cell division entirely.

There is no doubt that some substance is stimulated to activity under the influence of radium radiations which acts like a poison in that it produces the apparent abnormalities, and that this substance is intimately connected with the nucleus. But to call this substance a 'contagium vivo' and to endow it with the property of producing the observed results does not aid in the solution of the problem. The solution must be fundamentally chemical in nature, even though the precise reactions involved cannot now be discovered.

The following investigation was made to find how the early development of the egg is affected by radium radiations when (1) the sperm is exposed; (2) when the egg is exposed; and (3) when the egg is exposed immediately after insemination. The problem was suggested by Prof. T. H. Morgan, to whom I take pleasure in expressing my thanks.

METHODS

Nereis limbata was used in these experiments. In its sexual phase it can be obtained in great numbers during the summer months at Woods Hole, Massachusetts. This material has a great advantage in that practically 100 per cent of the eggs segment and develop normally under appropriate conditions, showing great regularity in the rate of development. The sexually mature individuals swarm in great numbers at the surface of the water on dark evenings, and can be kept, with proper precautions for many hours before they discharge their sexual products. For each experiment the eggs of two females were used. It was found necessary to work with small quantities of eggs since the jelly which the eggs extrude at the time of insemination is very mucilaginous and tends to mat together, in which event the eggs fail to develop normally. To prevent this it is necessary to stir them frequently during the first fifteen minutes, at the end of

which time the jelly loses its sticky character. The eggs were killed in Meves' modification of Flemming's fluid. The sections were cut 5 μ in thickness and stained with iron hematoxylin.

THE NORMAL DEVELOPMENT OF NEREIS

The normal fertilization of *Nereis* has been described by Lilie ('12) so that an extended account here is unnecessary. My observations are in every respect in accord with his. The following is a greatly abridged history of the fertilization phenomena in which only the more important events are mentioned.

As soon as the successful spermatozoön is implanted in the egg membrane it loses its motility and remains exterior to the egg for about fifty minutes. Very shortly after its implantation the cortical zone of colloidal material in the egg begins to be extruded, forming a thick jelly which, as it flows out, pushes away all the spermatozoa except the implanted one. The surface of the egg just beneath the perforatorium of the sperm now rises up in the form of a cone (fig. 1). The fertilization cone thus formed soon sinks, drawing with it the sperm which consequently comes to lie in a depression of the vitelline membrane. The cone, in the meantime has taken a deep stain and is a conspicuous object at the periphery of the egg. About fifty minutes after insemination the cone begins to move inwardly toward the center of the egg pulling with it the sperm head, which, as a consequence of being drawn through the membrane, assumes a band-like appearance. (fig. 2). The middle piece and the tail are left behind. The cone, followed by the sperm head, now moves deeper into the egg and revolves through 180° so that the sperm head is in front. The cone is left behind at this stage while the sperm nucleus moves forward. At this time a sperm aster develops (fig. 3). Later this structure divides unequally, the two asters thus arising forming the cleavage asters. As the sperm head advances the chromatin becomes vacuolated and later breaks up into the haploid number of karyomeres. These gradually fuse, the chromatin spinning out into a delicate spireme. At this time the sperm nucleus is ready to fuse with the egg nucleus (fig. 4).

In the meantime, the polar bodies have been extruded and the egg nucleus, at first very small after the running together of the vesicular chromosomes remaining after the second maturation division, now enlarges. The chromatin forms at first into karyomeres, just as in the case of the sperm nucleus, and then spins out into a delicate spireme. The two germ nuclei now fuse. The spiremes thicken and break up into chromosomes, which, after the first cleavage, become vesicular and finally run together forming the typical resting nucleus of the blastomeres.

The early cleavage needs no description, but a few typical stages that have been chosen arbitrarily for purposes of comparison with the abnormal larvae will be mentioned. In ten hours (depending somewhat on the temperature) the embryo acquires the protrochal band of cilia and begins to swim about actively. Green pigment at the posterior end develops in about fifteen to eighteen hours. In thirty five hours reddish brown pigment develops just back of the protrochal ciliary ring. A little later the first setae appear. At sixty hours the setae are in two pairs and soon after become jointed. The third pair also develops at about this time. One hundred hours after fertilization the palps are formed, and the whole embryo is markedly segmented.

EXPERIMENTAL

a. The fertilization of normal eggs by radiated spermatozoa

The spermatozoa of *Nereis* show remarkable vitality under very adverse conditions. If they are taken from a freshly caught, vigorous male and kept without admixture with sea water, they will remain alive for upwards of fifteen hours, and at the end of that time will fertilize fresh eggs in a normal manner, the embryos arising from such a union showing no marked abnormalities. They can live for a short time in practically fresh water, a fact that renders the sterilization of pipettes a troublesome process. If pure sperm be diluted with sea water the spermatozoa die within a few hours.

The fresh spermatozoa, as free from sea water as possible, were exposed by putting a small drop of the fluid in a thin walled glass

vial of such a size that it exactly fitted into the depression in the capsule in which the radium salt was held. The radium in this experiment was equal to 4 mg. of the pure bromide. The drop of spermatozoa was so spread out on the bottom of the vial that it formed a thin layer, all parts of which must have received equal radiation. A control experiment was made by putting another small drop into a similar vial which was placed near the other, but screened from it by two sheets of lead. As a rule, the spermatozoa were radiated for at least twelve hours, for it was found that shorter exposures produced very slight effects. After so long a treatment the motility of the spermatozoa seemed to be unchanged. Indeed they showed no signs whatever by which to distinguish them from the control or from perfectly fresh spermatozoa. These results are in accord with the statements of G. Hertwig ('12) in regard to the spermatozoa of sea urchins.

The phenomena of fertilization that can be observed in the living egg are normal. Great numbers of spermatozoa collect at the periphery of the eggs and can be seen to be pushed away by the gradual outflow of jelly which forms as soon as the successful spermatozoön is implanted in the egg membrane. If india ink is added to the water it becomes evident that no jelly is extruded at the point of attachment, the spermatozoön appearing to lie at the apex of a cone of ink which marks the region where the jelly is lacking. The time elapsing between the addition of the sperm and the outflow of jelly appears to be normal. Soon after the implantation of the spermatozoön the fertilization cone develops, reaching up to the surface of the egg membrane just below the point of attachment. The further progress of the fertilization phenomena can be seen best in sections.

Eggs killed thirty minutes after insemination show the spermatozoön, entirely normal in appearance, still external to the egg, with the perforatorium extending through the egg and into the substance of the fertilization cone. A little later the cone is retracted, pulling with it the sperm head and egg membrane so that the latter forms a small depression in which the former lies. Then the sperm head begins to be drawn out, penetrating further into the cone, and developing about its perforatorium.

the usual attachment granules. The cone itself develops normally, and its behavior in drawing in the spermatozoön is normal.

When the sperm head is in the process of being drawn in by the fertilization cone the first abnormality makes its appearance. The chromatin of the head, instead of forming an unbroken band, as in the normal process may now become broken up into irregular masses (fig. 6). Occasionally, in the normal sperm head as it is drawn through the egg membrane, one finds some evidence of segmentation, but in no case is there so marked a breaking up of the chromatin as is found here. About 10 per cent of the eggs show this abnormality.

From this stage the subsequent behavior of the sperm heads may differ. The further entrance and development of the sperm nucleus may be normal, or the sperm may entirely fail to gain further entrance. In the first case, the sperm head, preceded by the fertilization cone, penetrates further into the egg, revolving meanwhile. After complete revolution the sperm aster develops. The division of the sperm centrosome was found in only a few instances, but was normal when found. The chromatin becomes vacuolated and later forms into the haploid number of karyomeres. At this stage it may normally fuse with the egg nucleus, or else undergo a curious development which results in its failure to fuse with the latter. When fusion occurs the cleavage nucleus divides normally with twenty-eight chromosomes present. There is, then, either a complete fusion with subsequent normal division, or else no fusion at all, and no division of either germ nucleus.

In the latter instances the germ nuclei remain in the karyomere stage. The sperm asters which at first are present now gradually dwindle and finally disappear altogether. Figure 7 illustrates this point. The two asters are hardly as large as the smaller of the original sperm asters. At this time the egg aster has normally disappeared. This case and others to be described are not due to polyspermy, for such eggs are readily recognizable. The number of karyomeres present also shows that only one sperm has entered. Figure 8 shows an egg in which no trace of astral radiations can be found. There are many such eggs in the preparations. The action of the radiation on the sperm, then, has been to prevent

not only its own development, but that of the egg nucleus also. The number of vesicles and karyomeres differ considerably. Normally there should be at most four or five vesicles with twenty-eight karyomeres. Frequently the number of vesicles may be as high as twenty. The appearance of such eggs recalls the condition described by Lillie ('02) in the fertilized egg of *Chaetopterus* which had been treated with KCl solutions of varying concentrations. He found that the nucleus broke up into many fragments which were distributed irregularly through the protoplasm, and were never gathered up into a single nucleus again. Each particle of chromatin was surrounded by a vacuole of liquified protoplasm. The appearance is similar in many respects to that found in *Nereis*, although the mode of formation of the vacuoles is entirely different. Whether both of the germ nuclei are concerned in this process or only one, cannot be definitely settled. The presence of no extra asters shows that if the sperm nucleus has divided the chromatin only has been concerned. In view of G. Hertwig's experiments on the sea urchin, in which the egg nucleus divided without fusing with the sperm nucleus, it seems reasonable to suppose that in *Nereis* also the egg nucleus divides by itself. But here the division is very abnormal, being due to something evidently brought in by the sperm.

The second class of abnormalities is caused by the failure of the sperm nucleus to enter the egg. There is always a fairly large number of abnormalities of this type in material treated in the way indicated, and each egg presents practically the same appearance. It should be mentioned at the outset that this condition is found, though rarely, in the controls.

Although the spermatozoa that have been radiated are as active as those in the controls yet some of them fail to effect a complete entrance into the egg. The actual attachment of the spermatozoön is undoubtedly due to its own activity, but its subsequent entrance into the protoplasm of the egg is due to the activity of the egg itself. It may be inferred, therefore, that the sperm does not, in these cases, call out the proper stimulus for the complete reaction. The egg is able to draw the sperm head in for a short distance only. The details of the entrance of such a sperm are

as follows: The moment the perforatorium becomes implanted in the egg membrane there occurs the usual outflow of jelly. Beneath the perforatorium there forms the usual darkly staining area of protoplasm. The fertilization cone rises to meet the point of attachment, then sinks, pulling the sperm head with it. But as the chromatin is pulled out it is seen to be broken up into irregular masses, a condition not seen in the controls. In some instances the band appears to be continuous, but arranged in a bead like fashion. Thus far, then, the effect of the spermatozoön on the egg has been merely to fail to elicit the full response by which it should be engulfed in the egg.

During this period the egg gives off the two polar bodies in a normal manner. But when the egg nucleus begins its reconstruction, it fails to develop in the usual way. The chromosomes increase somewhat in size, and each becomes surrounded by a deeply staining, granular matrix. The chromatin appears normal. The matrix stains about as deeply as the fertilization cone. Each chromosome, surrounded by the matrix, lies in a clear, non-staining vacuole. Occasionally they can be seen to be segmented, a condition not seen in the control. In such eggs there is a total absence of astral radiations. The egg aster disappears very early, and the sperm aster never develops because the sperm is still entirely exterior to the egg.

The development of the egg nucleus in such a manner is not due to any poison injected into the egg by the radiated spermatozoön, but rather to the failure of the sperm head to enter. That this is true may be inferred from the experiments on partial fertilization made by Lillie ('12). These consisted of centrifuging the fertilized egg at such times that the attached spermatozoön, still external to the egg membrane, was removed wholly or in part. In the former case, when no chromatin was introduced, the eggs extruded the jelly and extruded both polar bodies. But the egg nucleus, instead of forming a typical vesicle with karyomeres, failed entirely to develop, the chromosomes lying free in the protoplasm. Around each chromosome was a darkly staining matrix, evidently corresponding to the chromosomal vesicle, through no vesicular wall was formed. This condition is an

exact picture of that found in eggs in which the radiated spermatozoön has failed to enter.

It is evident, then, that the radiation of the spermatozoa is not responsible for the peculiar development of the egg nucleus. Nor is the failure of the sperm to set up an appropriate reaction in the egg which should ensure its entrance an effect of the radiations per se. For the same condition is occasionally found in the controls. Probably any treatment which changes the spermatozoa would bring about the same result.

To sum up the results of this experiment, it may be said that the abnormal spermatozoa can be divided into two classes, according to the reactions they initiate in the egg. Those of the first class are able to activate the egg normally and are consequently drawn into the egg protoplasm; those of the second class are able to produce this result only in part. The spermatozoa of the first class induce the normal extrusion of the jelly, and the formation of the fertilization cone, but they fail to develop the normal cleavage asters and fail to fuse with the egg nucleus. The second class of spermatozoa bring about the normal extrusion of the jelly, and the formation of the fertilization cone, but cannot stimulate the egg sufficiently to cause it to draw them in. This defect results in the abnormal development of the egg nucleus. It cannot be said that these spermatozoa are injured in the chromatin alone unless it is held that any process that weakens their vitality acts specifically on the chromatin. It is apparent, however, that the chromatin is actually injured. This experiment shows that when failure of the nuclei to fuse occurs, the egg does not develop at all, thus differing radically from the sea urchin egg under similar conditions.

b. The subsequent development of eggs fertilized by radiated sperm

Cell division in the majority of eggs is normal, although usually somewhat delayed. The chromosomes split in the normal manner and form vesicles at the telophase. Later the vesicles fuse to form the resting nucleus. No irregular mitoses were

found. Those eggs in which the germ nuclei fail to fuse do not divide.

The first striking abnormality in the growing embryos appears when they are about twenty hours old. At this time the pro-trochal band of cilia and the green pigment at the posterior end are wholly lacking. Such embryos are of normal shape, but remain motionless, while the others in the dish are swimming actively about. In twenty-eight hours some are still lacking in ciliation, while others have developed the ciliary ring, which shows characteristic abnormalities in the distribution of the cilia. Instead of being in a continuous band, they occur in patches scattered irregularly. Such embryos swim slowly, and with a curious uneven motion, pursuing a devious course. In forty-eight hours, when the control embryos are well provided with green and red pigment and swimming vigorously at the surface, the radium embryos are still unpigmented, or else have the pigment broken up into small patches. The ciliation is still abnormal. The motion is consequently abnormal, the embryos tumbling over and over, or taking a spiral course, turning on their long axis. In seventy-eight hours a few of the embryos develop setae and pigment, but both are abnormal. The setae rarely grow out to full length, and are irregularly distributed. The majority of the embryos that have survived as long as this remain in about the same condition that they showed at thirty hours.

This mode of development occurs occasionally in the controls, especially if the conditions of growth are unfavorable, but never in as large proportions as among the radium embryos. Unfavorable conditions obtain if many eggs are allowed to develop in a small amount of water, or if they become matted together after fertilization. If eggs are compressed under a coverglass during early cleavage they develop precisely the same type of abnormalities.

In these embryos the effect of the injured spermatozoön remains latent for many hours, and is displayed for the first time when the embryo begins to differentiate the pigment and the cilia, and begins to grow notably in size.

c. The development of eggs radiated before fertilization with fresh sperm

In the second series of experiments the unfertilized eggs were placed in a few drops of water in a watch glass. The glass tube containing the radium was about 1 cm. in diameter and 1.5 cm. in length. When this tube was set down into the water, the surface tension of the water was sufficient to draw the eggs up along the sides of the tube, thus distributing them fairly evenly, and ensuring an equal amount of radiation. Control eggs were treated in the same way except that the glass capsule, similar to the radium capsule was empty. In all of these experiments small quantities of eggs were used.

The unfertilized eggs, after two hours exposure to the radium rays show no change in appearance. The germinal vesicle and the alveolar layer are intact. High magnification shows no alteration in the protoplasm, or in the oil and yolk spheres. When such eggs are inseminated with fresh sperm many immediately throw off the jelly in a normal fashion. As a consequence the alveolar layer quickly disappears. The jelly is apparently normal in consistency, and loses its mucilaginous character in about fifteen minutes, just as with normal eggs.

A considerable number of eggs, however, fail to extrude the jelly at once and consequently cannot push away the great quantity of spermatozoa that surround them, as can the normal eggs. Polyspermy results in such cases, many eggs being penetrated by a dozen sperm heads. Sections of such eggs show the alveolar layer still intact (fig. 9). This layer presents a fairly normal appearance, with alveoli closely crowded together, but not always radially arranged. Typically it extends entirely around the egg, and is about six or seven μ in diameter. But in these eggs it is very unevenly distributed, being sometimes absent altogether from one side, and massed in a thick layer on the other. There is no definite place at which the layer collects in these cases.

The eggs showing this peculiarity are uniformly larger than normal, the increased diameter being due to the greater width of the alveolar layer which varies from eight to fifteen μ in thickness.

When massed at one point it exceeds this limit. The explanation for this phenomenon is not known. According to Lillie, the extrusion of the jelly takes place when the spermatozoon becomes implanted. As the jelly diffuses outward sea water passes in to take its place, so that the original size of the egg is maintained. If this is true, it may be that the sea water has entered faster than the jelly has been extruded, and consequently, the whole layer increases in width. However, this does not explain the fact that the alveolar layer is unevenly distributed.

The increased size of the cortical layer does not persist as measurements show. Within seventy minutes after fertilization it has decreased noticeably, and after eighty minutes has disappeared altogether. The following figures are average measurements of a large number of eggs.

	<i>Normal</i>	<i>Radiated</i>
45 minutes after insemination . . .	106 \times 92 μ	115 \times 105 μ
60 minutes after insemination	105 \times 87 μ	111 \times 95 μ
70 minutes after insemination . . .	105 \times 90 μ	107 \times 95 μ
80 minutes after insemination	104 \times 91 μ	105 \times 94 μ

It is thus seen that before the first division, which occurs in about ninety minutes, the eggs are of normal size. Sections show that the alveolar layer by that time has entirely disappeared, except in the few cases in which fertilization did not occur.

In the control series the alveolar layer is extruded at once and nothing is seen of it inside the egg forty-five minutes after insemination. The germinal vesicle also breaks down soon after the extrusion of the jelly. I found no exception to this. But in the radiated eggs the vesicle shows some curious modifications in behavior. It may break down at once even though the alveolar layer is still present, or it may remain intact for a considerable period. In the former case, the chromatin, which, during the process of dissolution of the nuclear membrane has collected in the form of chromosomes, is left free in the protoplasm. If the vesicle remains entire, the chromatin, which is now in granules, collects along the periphery, just beneath the membrane. This is the normal condition for it at this period. Occasionally the granules collect in the form of chromosomes which are scattered

through the nucleus suspended in a delicate, non-staining network. After a time (varying in different eggs from forty to eighty minutes) the vesicle breaks down, and the subsequent history of the chromatin is the same as in the case of eggs in which it disappeared at once. Its dissolution is apparently independent of the presence of the alveolar layer. Here, then, is a case in which the chromatin is not visibly affected by the radium radiations but the protoplasmic activities are much modified, although the structure of the protoplasm itself remains unchanged in appearance. This fact is in opposition to Hertwig's view that only the chromatin elements are affected by the rays.

The mode of entrance of the spermatozoon into eggs which retain their alveolar layer is considerably modified from the normal. The fertilization cone rises up under the point of attachment of the perforatorium as usual, and later sinks into the egg, pulling the sperm head with it. But it takes no stain at all. Figure 12 shows an egg killed forty-five minutes after fertilization. The spermatozoon has become implanted in the egg membrane, and has begun to penetrate a short distance into the alveolar layer. No cone is seen. A later stage is shown in figure 10 in which the alveolar layer is still intact and is wider than normal. The sperm heads have penetrated nearly their entire length. They never completely enter the protoplasmic area of the egg, but remain in the alveolar layer until it is extruded, at which time some of them continue their course inward. Intermediate stages in this process have not been found. It may be inferred that the sperms enter entirely since at the time that the cortical layer is extruded (in about eighty minutes) none can be found exterior to the egg, while within many may be found in various stages of development.

The process of maturation in those eggs which fail to extrude the alveolar layer at once is much modified. The germinal vesicle disappears normally in the great majority of cases. The chromatin, now free in the protoplasm, may move toward the animal pole where the spindle normally should form, or it may remain in the interior of the egg. Figure 11 shows a case in point. The chromosomes are ill-formed, show no particular arrangement

and are deep in the egg. Careful search has not revealed any asters or spindle fibers. In figure 12 the spindles have formed, as well as the chromosomes, but the asters are lacking. Here, too, the spindle forms in the center of the egg. There are other cases in which very small asters are present, with fairly normal chromosomes. Occasionally figures like figure 13 occur, in which the achromatic portion of the figure is normal, but the chromosomes are segmented. A more normal type is seen in figure 14. The normal condition seems to be due to the fact that the alveolar layer has been extruded at the periphery nearest to the spindle, allowing the usual relation to obtain between the protoplasm and the sea water in the egg protoplasm.

In a majority of cases, however, the alveolar layer is extruded normally. In all instances the spermatozoa may enter normally or may show a characteristic abnormality, which, curiously enough, is similar to a condition found in normal eggs fertilized by radiated sperm. It consists in the failure of the fertilization cone to stain the usual dark color. Figure 15 shows such a condition. It will be noticed that the sperm is normally implanted and has already become anchored in the substance of the cone by minute attachment granules. The cone itself is transparent, and traversed by a delicate achromatic network. Immediately around it the protoplasm is of normal appearance. Later the cone sinks into the protoplasm in the normal manner, pulling with it the sperm head. The non-staining quality apparently has no effect on its function.

Lillie suggests that the normal deep stain of the cone is due to a coagulating fluid injected by the sperm into the egg through the perforatorium. If this is true we have another evidence that the chemical reactions of the egg protoplasm, have been changed by the radiation.

The formation of the first polar body may be very abnormal, but the abnormalities concern chiefly the achromatic portions of the spindle. As a rule the chromatin is but slightly affected. Figure 16 shows a well marked tri-aster with large centrosomes which are abnormal in size. It will be noticed that the chromosomes, even in the minutest details of splitting are perfectly nor-

mal. This condition can hardly be interpreted as a first and second polar division combined, since it occurs much earlier than the normal time for the latter. The distribution of the chromosomes shows also that the two lower asters do not represent the second spindle. The sperm is still exterior. Figure 17 is more normal, having but two asters. The spindle has not revolved, as it should have done at this time. The normal figures are at the telophase of the first polar spindle. The asters are of normal size and appearance, and the chromosomes show no abnormality of any sort. It is curious that they have taken up a position outside of the spindle, more or less in the place which they should occupy if the spindle had revolved.

There are many other abnormalities in the formation of the spindle, but the examples given are typical. In practically no case in which the spindle forms do the chromosomes show any abnormality.

The second polar spindle may be entirely suppressed or may develop abnormally in several respects. When the first polar body is not extruded, the second also may fail to be given off. Figure 18 shows what is evidently a second maturation. The egg was killed eighty minutes after insemination, at a time when the control eggs **had** already extruded the second polar body, and possessed a well developed egg nucleus. The spindles are normal in general appearance, but abnormally small, and placed in the center of the egg. It is difficult to say what would be the future history of such an egg. Evidently the polar bodies will not be extruded. Figure 19 shows a similar case except that the spindles are larger. It will be noticed that one of the sperm asters has formed before the revolution of the sperm head and cone, an unusual occurrence.

It is evident, then, that the injury done by the radium has affected both the chromatin and the protoplasm, particularly the latter. When the alveolar layer is not extruded the astral systems are abnormal and consequently the chromosomes. In such cases it is difficult to say how far the chromatin has been injured.

After the maturation period the remaining chromatin collects in one or more large vesicles of normal appearance. Occasionally the number of such vesicles may be very large and the karyomeres very numerous. The extra number may be due to the fact that the polar bodies have not been extruded, so that there is much extra chromatin. The subsequent behavior of these vesicles differs accordingly to whether they fuse with the sperm nucleus or not. In the former case the fusion may be entirely normal, and the subsequent division perfectly regular. Or the cleavage nucleus may never divide, but, on the contrary, increase considerably in size until it is nearly half as large as the germinal vesicle. The chromatin, in the meantime, breaks up into minute granules and passes out from the nucleus into the surrounding protoplasm. Figure 20 shows such an instance. The nuclear wall displays no breaks through which the granules might have passed out bodily.

Such a condition might be explained on the basis that the egg chromatin had been injured by the radiations and had formed, as a result, a poison, which affects the sperm chromatin. The general effect of the radiation is to cause the chromatin to break up into granules. This is the explanation suggested by G. Hertwig.

If the sperm nucleus does not fuse with the egg nucleus it fails to develop past the spireme stage, and in many cases does not develop even as far as that. Up to this time it has behaved normally. Even when many sperms enter (fig. 19) each one pursues the normal course in the egg. In figure 21 are shown two sperm nuclei in several separate vesicles, in which the karyomeres have fused and commenced to draw out into spiremes. Which vesicles belong to the egg and which to the sperm nuclei is difficult to determine since they have become exactly the same in appearance. In such a case, if a poison has been generated, it has failed to produce any marked effect on the egg chromatin itself. The only apparent abnormality is the failure of the nuclei to fuse.

Division of the egg may occur without the appearance of asters and without division of the chromatin. Figure 22 shows an egg in which all the chromatin is in one blastomere. Figure 23 shows the protoplasm dividing without any division of the chromatin.

atin. The vesicles, which have not developed into the spireme stage, are scattered irregularly about in the protoplasm. The large number of vesicles, with forty-two karyomeres, and two asters, indicates that two sperm nuclei are present. But they have ceased to develop and their astral systems have failed to develop normally. This failure is evidently not due to polyspermy for polyspermic eggs develop in a different and characteristic manner.

To sum up: The effect of radium on the unfertilized eggs is seen after fertilization with normal sperm in both the protoplasm and the chromatin. The failure of the surface layer to be given off is due to a change in its constituents under the influence of radium. This failure leads to abnormalities in the formation of the polar bodies. The chromatin at first displays very little evidence of injury. Later, however, the injury shows in the failure of the egg nucleus to develop properly. The sperm nucleus also fails to develop in these cases.

d. The development of embryos from radiated eggs fertilized by normal sperm

The later development of these eggs is not different from that of the normal eggs fertilized by radiated sperm, but the proportion of unfertilized eggs is greater, and the rate of development is slower. This may be inferred from the fact that the sperm, in many cases, cannot enter the egg for more than an hour after insemination because the cortical layer is still present. Occasionally some eggs divide into three or four parts at once, or, if into two blastomeres, the relative sizes of the two are abnormal. The subsequent development is also greatly retarded. When the control embryos are already in the early trochophore stage, these have not yet acquired the protrochal ring of cilia. A large proportion die before they reach the trochophore stage. Whether the proportion of abnormalities is greater in this series than in the first is hard to say, since always in the controls, a few embryos are abnormal. The type of abnormality that develops is precisely the same as that described in the preceding section.

c. The development of eggs normally fertilized, and exposed to radium

In this series of experiments, the eggs were inseminated and stirred frequently for five minutes, then put over the radium capsule, which had an activity equal to 4 mg. of the pure bromide. At intervals a small quantity was taken out, half of the eggs being killed, and half put into sea water and allowed to develop.

The early stages of fertilization are normal in all respects. The jelly is extruded at once (this began before the eggs were exposed to the radium) and consequently only one sperm enters. The external phenomena of implantation of the sperm and the rising up of the entrance cone are normal. A study of the sections shows that in many cases the fertilization cone does not take the usual dark stain, but remains as a very light, nearly transparent vesicle, similar to that described in the second series of experiments. (fig. 15). The sperm enters normally, however, as in the former case. In the meantime the polar bodies are normally extruded.

The first indication of abnormal development is seen in the behavior of the germ nuclei. In many instances these do not fuse, but remain separated, at some distance from each other and independently develop chromosomes and normal spindles. Figure 26 shows an early stage of the process. The nuclear walls are still intact. The sperm nucleus has not developed as far as the egg nucleus, being still in the closely wound spireme stage. The egg nucleus with its aster has moved from its position under the polar bodies, away from the approaching sperm nucleus. Figure 24 is a later development. Probably the structure nearer the polar body is the sperm nucleus which has failed to develop much further than the stage shown in the preceding figure. The egg nucleus, however, has lost its nuclear membrane and has formed distinct chromosomes which are not as clear cut as normal, but show some evidence of becoming broken up into granules. Figure 25 shows a still later development. The nuclei have migrated as far from each other as appears possible and lie between complete and normal asters. In each nucleus the haploid number of chromosomes can be determined. In figure 27 the division has

gone still further. Judging from the size of the chromosomes, it seems probable that they have split, fourteen daughter chromosomes going to each pole; but the number could not be exactly determined. The chromatin in this case is perfectly normal. In the instances mentioned, and in many others that have been observed, it is apparent that the plane of one spindle does not coincide with that of the other or with the first normal cleavage plane.

Occasionally, when the nuclei fail to fuse, the astral systems do not develop, or, if they do, remain only a short time. The chromatin in such cases degenerates, forming irregular, granular masses which lie free in the protoplasm (fig. 28). One germ nucleus has evidently degenerated faster than the other and its chromatin has become much scattered and takes the hematoxylin stain very lightly.

When the fusion of the nuclei occurs the process appears to be normal. Abnormalities appear when division begins. As a rule the chromatin shows few signs of injury, but the astral systems are abnormal. Very commonly a tri-polar figure arises (fig. 29). The asters are normal in appearance and the centrosomes are normal. The chromosomes are very irregularly arranged, as would be expected. But each one is well formed, showing no evidence of degeneration.

Frequently, however, the chromatin displays undoubted signs of injury, as shown in figure 30. Not only are the chromosomes indistinguishable but some of the strands are now broken up into minute granules. There are all gradations between such a condition and a normal figure of which there are very few in the material.

The effect, then, of the radium on fertilized eggs is much more marked than that on the unfertilized eggs. This accords with the results of O. and P. Hertwig, who found that the embryo frogs arising from radiated fertilized eggs showed more marked abnormalities than those in the other series. The effect on the chromatin are similar in some respects to those described by P. Hertwig in the egg of *Ascaris*. It is evident, however, that the protoplasmic activities of the eggs of *Nereis* are almost equally affected

with the chromatin, and that the fertilized eggs in the two cases respond in very different ways.

f. Subsequent development of the embryo

The great majority of the eggs do not divide. In some cases they may divide into three or four blastomeres at once, as would be expected from the division figures already described. Very few of the eggs continued to divide after ten hours of development. A small number of early trochophores developed but no pigment formed in them, and the cilia were abnormal. No embryos survived after twenty-four hours.

*g. Development of eggs radiated before and after fertilization
with normal sperm*

The early phenomena of fertilization in eggs radiated for two hours before fertilization and for varying periods *after fertilization*, do not differ to any extent from those described in the second series. In some lots of eggs the alveolar layer is extruded normally in almost every instance, while in other lots it is retained for some time after the implantation of the sperm. In the latter case polyspermy invariably occurs, as many as ten sperms entering at practically the same time and developing at the same rate.

The mode of entrance of the sperm in either case is the same as that described in the second series. In many instances the fertilization cone is an almost transparent vesicle. But, as in the previous cases, this change in the staining reaction does not indicate any change in function, for the spermatozoon is drawn in normally, and the whole process is without any other indication of abnormality. The further development of the sperm head is normal. The head and cone revolve, and an aster forms at the base of the head. The chromatin, as the head enlarges, collects into a few karyomeres. But the number of vesicles which contain these structures may be very large. In some eggs there may be as many as forty, indicating that each sperm has broken up into at least four. Their subsequent development will be described after the behavior of the egg nucleus has been traced.

The germinal vesicle breaks down as a rule, within a few minutes after insemination, even though the alveolar layer is still present. The egg centrosome divides normally, the daughter centrosomes migrating to the opposite sides of the group of chromosomes which lie free in the protoplasm without any definite arrangement. If the alveolar layer has been extruded the spindle usually moves to the surface where it develops normally in many cases. In such instances the chromosomes are not at all affected, but split into the normal crosses and rings with great precision. The achromatic figure also appears to be normal. When the alveolar layer is not extruded at once, and, in some cases, even when it is, the whole first polar figure remains near the center of the egg. Judging from the scarcity of extruded polar bodies, it is probable that the polar body is not often extruded.

There are, however, several marked departures from the course outlined above. Figure 31 shows a condition found in several eggs. There is but one centrosome and no real aster. The spindle fibers are normal and attached to the chromatin masses. No normal chromosomes are present, the chromatin remaining either as a long narrow thread, or as irregular, homogeneous masses. In each instance it is in two distinct groups separated by a varying distance. The size of the chromatin thread indicates that splitting has not occurred in the right hand group. In the other group the chromatin is in very irregular masses of unequal size. Figure 32 is a later stage of the same condition. In this egg there is a normal sperm head and cone already revolved, but the sperm aster is not apparent. Some of the chromosomes are fairly normal, while others are mere masses of chromatin. The separation of the groups and of the spindle fibers attached to them is a curious phenomenon. In some instances both polar bodies are given off, but in many cases none at all are extruded.

During this time the sperm heads have developed up to the vesicular stage, as has been mentioned above. Their further development may proceed along different paths. In some instances the karyomeres apparently break down and fuse together, taking the form of fine spireme, or even breaking up into chromosomes. If several sperm heads enter there are at least as many

asters, and in many cases, a much greater number. But such asters are not connected with each other by spindle fibers nor are they centers for chromatin masses. In some instances a great number of asters is present but their origin is uncertain. If we assume that each original sperm aster has divided normally, it would be necessary that fifteen sperms entered the egg about at the same time. This number is greatly in excess of any observed case. The extra asters may have arisen *de novo*.

Perhaps the most common abnormality is the presence of a great number of vesicles without any aster. In view of the facts just presented, namely, that sperm asters develop when polyspermy occurs, it is evident that the extra vesicles represent a single sperm nucleus that has divided, or else the egg nucleus, or finally, sperm nuclei that have lost their asters. Figure 33 is a case in point. The only aster present is extremely small. The vesicles, of which there are a great number in the egg, are still intact although the karyomeres have about disappeared, having gone into the spireme form. At this stage we should expect the germ nuclei to fuse but in this case, one hundred minutes after insemination, when the control eggs have already divided, we find no evidence of fusion. Such eggs do not divide but show the very common phenomenon of budding.

By many observers this phenomenon has been described in eggs that are rapidly degenerating. In the eggs of *Nereis*, at about the time when cleavage should take place, the protoplasm at the periphery separates off into buds of irregular size and shape. They contain no chromatin unless by chance some of the vesicles are in the region where the budding occurs. The protoplasm of the buds and the region directly contiguous shows marked signs of degeneration, appearing to be more fluid and possessing only a few protoplasmic granules, instead of the closely crowded quantities present in the more normal regions.

The general effect of the radium radiations on the eggs treated in this manner seems to be more on the protoplasm than on the nucleus. The surface layer is greatly affected and as a consequence may fail to extrude the alveolar layer. When division, would normally occur, only a budding takes place. If the sperm

asters are developed from the egg protoplasm, it is evident from their peculiar behavior that the latter substance has been injured. The chromatin also is affected, as is shown in the cases of irregular formation of the maturation chromosomes, and in its behavior in the nuclear vesicles. But the many cases of perfect formation of the chromosomes and their normal splitting, even in the minutest details, indicate that the effect of the radiations on the chromatin has not been profound.

DISCUSSION

The work of G. Hertwig and others points to the conclusion that the chromatic elements of the nucleus are the only parts of the cell which are disturbed by the radium radiations. The conclusion that no other constituents are affected has been drawn from the fact that the usual methods of technique do not reveal any chemical changes in the protoplasm. Such a criterion is, in many cases the best we have, but it is evident that staining will not reveal all of the cell constituents which are known to be present, which, like enzymes, operate on the protoplasm of the cell, changing its chemical constitution; nor will it give evidence of the nature of such changes.

There are no visible changes in the radiated sperm—a fact that led Hertwig to believe that the protoplasm was uninjured. But I believe that in the sperm of *Nereis* there is evidence that a protoplasmic change has taken place. This change becomes apparent through the activities of the egg in the early stages of fertilization—activities which are incited by the stimulus of the sperm itself. In the first place, when the sperm becomes implanted in the egg membrane it injects into the egg a coagulating substance which, according to Lillie, changes the protoplasm so that it takes a fairly deep stain with hematoxylin. This staining reaction is frequently lacking. It cannot be said that the egg protoplasm has been changed since all of the eggs used in the experiment were normal. Therefore it may be inferred that the protoplasmic elements in the sperm have been modified.

Secondly, the sperm in many instances fails to gain entrance into the egg. The engulfing of the sperm is undoubtedly due to the activity of the egg, since the sperm is motionless from the moment it becomes implanted in the egg membrane. Such activities are incited by some substance in the sperm itself, and are not due merely to the pricking of the membrane by the perforatorium, for in many instances the implanted sperm does not enter. The seat of such a stimulating substance is not certainly known. Probably it does not reside in the nucleus, since all of activities of the egg, such as the throwing off of the cortical layer, the formation of the fertilization cone, and its subsequent movements, occur while the nucleus is still intact and entirely outside of the egg. It is very likely that the stimulus resides in the head cap of the spermatozoön, and is injected into the egg with the coagulating fluid. The failure of the sperm to enter is due to the injury to this substance and a consequent failure properly to activate the egg. Such an injury is not due specifically to the radium treatment since old spermatozoa, kept fourteen hours in a vial will produce the same results. Probably any treatment which weakens them in any way has this effect. These two instances, I believe, indicate that the sperm protoplasm has been injured by the radium radiations.

That the chromatin of the sperm has been injured has already been pointed out. The difference in behavior between the *Nereis* sperm and that of the sea urchin, as described by Hertwig, should be mentioned. In the former case, the sperm nucleus fails to develop past a certain point, and fails to fuse with the egg nucleus. The latter structure also fails to develop due apparently to the failure of the sperm centrosome. In the sea urchin, on the other hand, the sperm centrosome develops perfectly, becoming the dynamic center of division for the egg nucleus, which divides by itself as a rule in a normal manner. If the sperm nucleus has migrated into a position close to the spindle it acts as a foreign body in disturbing the normal behavior of the chromosomes. In some cases the two nuclei fuse, but the sperm nucleus is soon eliminated in subsequent divi-

sions. Development is consequently parthenogenetic, a condition which does not obtain in the *Nereis* egg. It should be noted that the sea urchin egg is an especially favorable subject for parthenogenetic development, while *Nereis* is not. Consequently the two cases are not exactly parallel and the behavior of the one should hardly be expected to be repeated in the other.

The generally accepted theory that the cleavage centrosomes are derived from the divided centrosomes brought in with the sperm has recently been questioned by Lillie who believes that in *Nereis* the centrosome appearing at the base of the sperm head is derived through the interaction of the sperm chromatin and the egg protoplasm. The fact that the middle piece is always left behind on the outside of the egg membrane seems to support this view. A discussion of the theory is not in place here, but it may be pointed out in passing that this fact alone is not conclusive evidence that no formed centrosome has been introduced by the sperm, since in many insect spermatozoa the centrosome is contained in the sperm head. If, however, in *Nereis*, the centrosome is a product of the sperm chromatin and the egg protoplasm we have evidence that the sperm nucleus has been injured. If, on the other hand, the older view as to its origin is true we have evidence that the radium radiations have affected a protoplasmic structure.

A discussion of the effect of radiations on the unfertilized egg raises some interesting questions concerning certain protoplasmic activities of the egg. It has been shown that in many cases the cortical zone, composed largely of colloidal material is not extruded at the time of implantation of the sperm, but remains for a considerable period within the egg, and is given off slowly during subsequent development before cleavage. As a result polyspermy occurs. The extrusion of the surface layer is due, according to R. S. Lillie, to the sudden increase of permeability of the plasma membrane, resulting in an outflow of substances previously impermeable to it. That the membrane is rendered somewhat permeable at the time of implantation of the sperm is evident from the fact that water is taken up, resulting in a swelling of the alveoli. But the normal permeability is

not fully attained. The failure of the cortical layer to be given off may be due to a change in the plasma membrane, or to a change in the colloids of that layer. The possibility of such a change will be discussed later.

The formation of the polar bodies has been shown to be abnormal both in respect to the chromatic and achromatic elements of the spindle. It might be concluded from this that both parts were equally affected by the radium radiations but this is not necessarily true, for it has been shown that the presence of the cortical layer has an inhibiting effect on mitotic phenomena. This suggestion has been made by Bataillon who holds that in the cortical layer are certain katabolic products of metabolism which hold the egg in check until they are given off. The increased permeability at the time of fertilization allows them to be extruded. The resulting egg membrane is therefore a result of such an action and is incidental, indicating merely that certain more fundamental phenomena have been taking place. In *Nereis*, when the cortical layer persists, the maturation divisions are always abnormal, remaining permanently in the interior of the egg or else showing other defects which have been described above. If, however, the cortical layer is extruded in part, the spindle migrates to that region and the figure is normal. It should be mentioned, however, that occasionally in eggs from which the cortical layer has been extruded, the same type of abnormalities may be found. The radium radiations therefore, have affected not only the surface layer, but also the deeper lying protoplasm.

That the abnormalities in the chromatin appear during the formation of the chromosomes, when they are being built up from the protoplasmic constituents indicates that the latter themselves may have been modified, or that the agents through which they are so built are affected, as well as the chromatin itself. These abnormalities are even more strikingly manifested in the division of the fertilized egg, as described above, and also by P. Hertwig.

If we attempt to explain the phenomena already described by the hypotheses of Schwarz or of Hertwig we are at once involved

in difficulties. The latter involves a number of difficult assumptions. First, when the sperm is radiated, the poison generated in the nucleus must remain altogether in that structure, since the protoplasmic activity concerned in movement is not affected, or else it must be assumed that the poison is specific for the chromatin itself, not affecting the protoplasm. And secondly, it must exert no influence on the egg protoplasm during division, since a normal haploid division can occur provided the sperm head does not mechanically interfere in the spindle. If the sperm head fuses with the nucleus its poison exerts no influence on the egg chromatin, other than to cause it to eliminate the sperm chromatin. But it has been shown that in *Nereis* the protoplasm of the sperm has evidently been changed. Hence it would be necessary to postulate that the poison had extended into the protoplasm and had affected the sperm centrosome—a condition contrary to the first supposition. In the egg it is very evident that the protoplasm has been affected. Hence the hypothesis that poison is generated solely in the nucleus is not tenable. A modification of the poison theory will, I believe, explain more of the facts, and such a modification will be discussed later.

The lecithin hypothesis is hardly as satisfactory as the other. The fact that the sperm nucleus is undoubtedly affected by the radiations makes it evident that they are not specific for the lecithin in the protoplasm. The behavior of the radiated egg is more easily explained for it has been shown that the cortical layer, which contains considerable lecithin is affected. If that substance which is distributed throughout the egg is utilized in the upbuilding of the chromatin, the phenomenon could be explained.

These two hypotheses, therefore, fail at certain points when they are used to explain the facts observed in the fertilization of the egg of *Nereis*. I believe that I have given good evidence that both hypotheses may be true in part, since both protoplasm and chromatin are affected by the radium radiations. The question now arises as to the means by which such changes can be brought about.

The solution of the problem lies, I believe, in the fact that the protoplasmic and nuclear elements are not directly affected by the radiations but only indirectly by means of enzymes which are activated by the treatment. In support of this view may be mentioned a number of observations. The experiments of Schwarz ('03) and of Wohlgemuth ('04) prove that lecithin is readily decomposed by treatment with radium. But the latter observer, who investigated the action of radiations on pure organic substances such as olive oil, asparagin, peptone, and finally lecithin, found entirely negative results. In no case were there any observable changes in the substances. From this he concluded that the breakdown of the cells and tissues which contain these substances was due, not to the direct effect on them of the radiations, but to the increased autolytic activity of enzymes in the cells.

The question whether all cell constituents (nucleo proteids, simple proteids, lipins, carbohydrates, and salts) are affected cannot be determined, but an experiment by Wohlgemuth, the results of which are significant in this connection, throws some light on the subject. To test further his hypotheses that the autolytic enzymes are activated by the radium radiations, he exposed portions of tuberculous lung for varying periods, and found that the total amount of nitrogen given off was at first increased fourfold. In the end, however, the control material showed the same total amount as the other portion. But the experiment indicated that the enzymes which bring about a decomposition of the nitrogenous material in the cell are greatly activated. We should look therefore, to the proteins and to lecithin for evidences of decomposition.

That enzymes are activated by radium radiations has been shown by numerous observers. They exert an accelerating effect on peptic digestion, on the diastatic enzymes of the blood, liver, saliva, and pancreas. "This favorable action is not always observable immediately; very often retardation occurs during the first twenty-four hours, this being gradually neutralized, and then replaced, if the experiment is sufficiently prolonged, by an acceleration" (Euler). The promotion of plant growth is also

attributed by Falta and Schwarz ('11) to enzyme activation. Gager ('08) and Congdon ('12) have also found some evidence of acceleration in growth, if the intensity of the radiation is not too great. When *Drosophila* eggs are placed close to a strong preparation of radium, their development is retarded. From these experiments it may be concluded that many kinds of enzymes may be activated but that the lytic enzymes are more stimulated than those that play a synthesizing rôle.

The decomposition of the nitrogenous compounds of the cell is in part normal and in part abnormal. Lecithin breaks down into cholin and finally into trimethyl amine, a reaction which does not take place in life. But the decomposition of the nucleoproteids occurs constantly under normal conditions. Under the influence of enzymes these substances are oxidized to nucleic acid and finally into some of the purin bases (guanine, hypoxanthine, etc.). The reverse reaction also occurs, by which the complex nucleoproteid is built up again from the simpler materials. The seat of this process is at the nuclear wall, where the nucleoproteids and the protoplasmic proteins adjoin. If, then, we assume that under the stimulus of the radiation the katabolic changes in the nucleoproteids takes place at a more rapid rate than the synthesizing reactions (an assumption that is warranted on the basis of Wohlgemuth's results), we have an explanation of the behavior of the radiated chromatin in breaking up into granules, and failing to divide normally. The acceleration in growth, found by Gager and Congdon when the material was exposed to slight radiation, may be explained on the ground that under such conditions the synthetic processes are stimulated, while under a more intense radiation the opposite reaction obtains.

The behavior of the radiated cells must, I believe, be interpreted in the light of the increased activity of these autolytic enzymes which act both on the chromatin and on the protoplasm. A radiated spermatozoön is affected chiefly in its nuclear constituents which are broken down into simpler compounds. That these are still acid is indicated by their staining reactions. When such a cell enters the egg, the nucleus is unable to develop normally, and to divide with the egg nucleus, because it cannot build up

nucleo-proteids at a normal rate. Hence it is either eliminated, as Hertwig has shown, or remains stationary, after developing up to a certain point. In like manner, the egg nucleus, after the egg has been radiated, fails to develop. That the normal sperm which fertilizes such an egg may develop, as suggested by Hertwig, indicates that the egg protoplasm, made up of "simple" proteins, has not been greatly changed. In *Nereis* evidently such proteins have been affected, as shown by the abnormal behavior of the sperm nucleus. When the fertilized eggs are radiated, the radiation is applied at a period when the synthetic process in the formation of the chromosomes is most active. Hence, any acceleration of the autolytic enzymes results in a speedy deterioration of the chromatin. The latent period which is found in many instances when the radiation is neither prolonged or intense may be explained by this hypothesis. Only a slight acceleration is brought about by the treatment, but the effect of autolysis is cumulative, and becomes manifest after a period, depending on the rate of acceleration. The nuclear material has been broken down to such an extent that, after division, the chromosomes cannot be built up again. In this connection it might be suggested that the normal nuclear division takes place when the ratio between the amount of nucleoprotein and simple proteins of the protoplasm has reached a certain limit. After the division the ratio is changed and constructive action again takes place.

SUMMARY AND CONCLUSION

1. Radiated spermatozoa of *Nereis* react in two ways to the normal egg. They may normally stimulate it, and be drawn in, but subsequently fail to develop, or they may fail to stimulate the egg sufficiently and so remain external. In the first case, the sperm nucleus and aster may fail to develop and to fuse with the egg nucleus. In the second case, the egg nucleus develops without an aster.

2. The radiated egg at the time of fertilization may or may not extrude the cortical layer. In either case, the maturation phenomena are more or less abnormal. The germ nuclei develop

abnormally and mitosis does not occur, although the protoplasm may divide.

3. Radiation of the fertilized egg results either in a failure of the fully developed germ nuclei to fuse, or in abnormal division of the cleavage nucleus.

4. Eggs radiated before and after fertilization show very marked evidences of protoplasmic degeneration.

5. In general, both protoplasm and chromatin are affected.

6. The previous hypotheses do not satisfactorily explain these facts.

7. It is suggested that the radium radiations act indirectly on the chromatin and protoplasm by activating autolytic enzymes which bring about a degeneration of the complex proteids, and probably by affecting other protoplasmic substances in the same manner.

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All the drawings were made with a camera, with a 2 mm. Zeiss apochromatic objective, and a No. 6 compensating ocular, unless otherwise stated.

PLATE 1

EXPLANATION OF FIGURES

1 to 4 Normal Fertilization.

1 Attachment of the sperm; 55 minutes after insemination.

2 Penetration of the sperm head; 60 minutes after insemination.

3 Telophase of the second maturation division, and approach of the sperm nucleus; 65 minutes after insemination.

4 Just before the fusion of the germ nuclei; 90 minutes after insemination.

5 to 9 Fertilization of normal eggs by radiated sperm.

5 Penetration of the sperm head, showing absence of the fertilization cone; 60 minutes after insemination.

6 Development of the egg nucleus, and failure of the sperm nucleus to enter; 115 minutes after insemination.

7 Gradual disappearance of the sperm aster; 105 minutes after insemination.

8 Total disappearance of the sperm aster. The sperm nucleus has divided into many vesicles; 115 minutes after insemination.

9 to 29 Fertilization of the radiated eggs by normal sperm.

9 Polyspermy. This egg has failed to extrude the alveolar layer. 2 mm. apochr. and No. 0 eyepiece.

10 Detail of the same; 70 minutes after insemination.

11 Formation after the first maturation figure without astral system.

12 Same, with spindle but no asters; 45 minutes after insemination.

13 Same. The chromatin is segmented, but is splitting longitudinally 45; minutes after insemination.

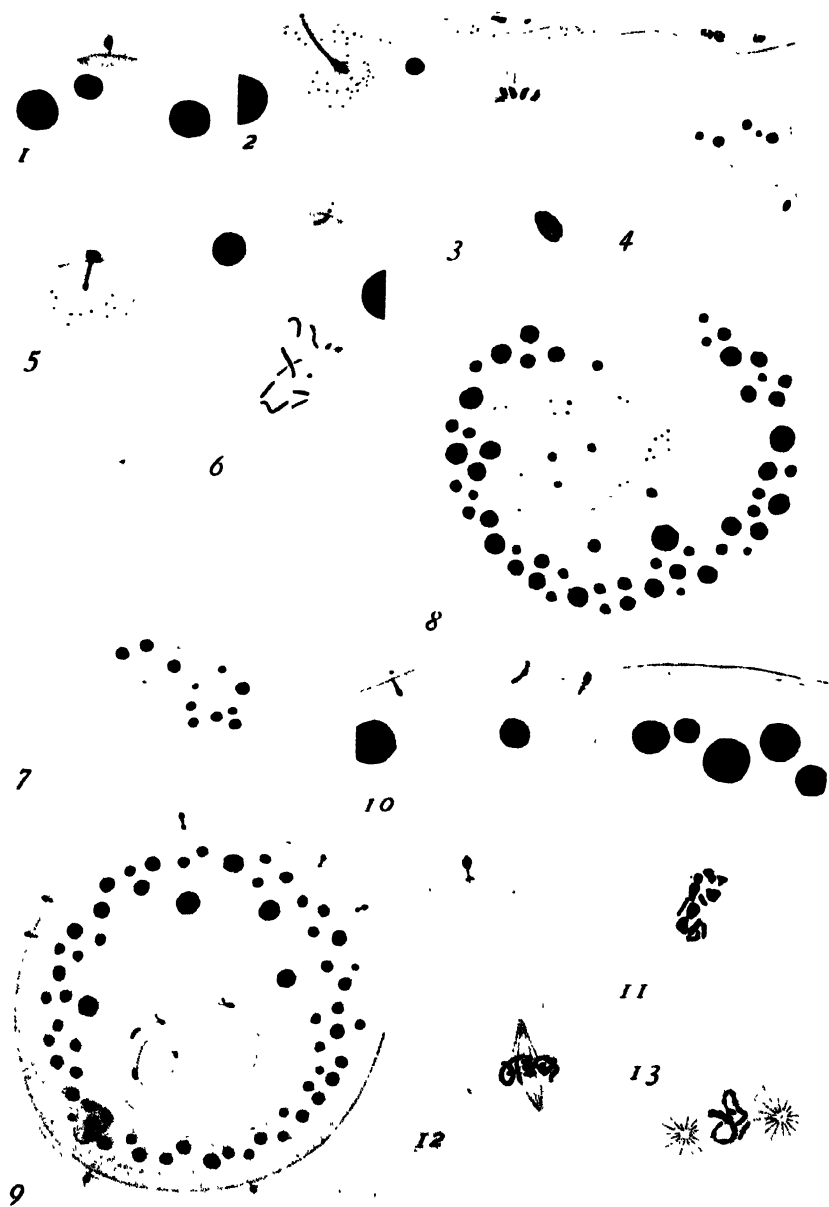


PLATE 2

EXPLANATION OF FIGURES

14 First maturation division occurring at a point where the alveolar layer has been extruded; 60 minutes after insemination.

15 Entrance of the sperm, showing the non-staining vacuole.

16 Tripolar spindle of the first maturation; 55 minutes after insemination.

17 First maturation spindle, showing an abnormal distribution of the chromosomes; 60 minutes after insemination.

18 The second maturation. The first polar body has not been extruded; 80 minutes after insemination.

19 The same; two sperms are entering in one, the aster has appeared before the revolution of the cone. 2 mm. X No. 0.

20 The cleavage nucleus. The chromatin is passing out through the nuclear wall; 120 minutes after insemination.

21 Polyspermy; 70 minutes after insemination.

22 Division of the protoplasm without a distribution of the chromatin. 2 mm. apochr. X No. 0 eyepiece.

23 The same; the same magnification.

24 to 30 Normally fertilized eggs radiated.

24 Approach of the germ nuclei; 105 minutes after insemination.

25 The nuclei have migrated to opposite sides of the egg; 105 minutes after insemination.

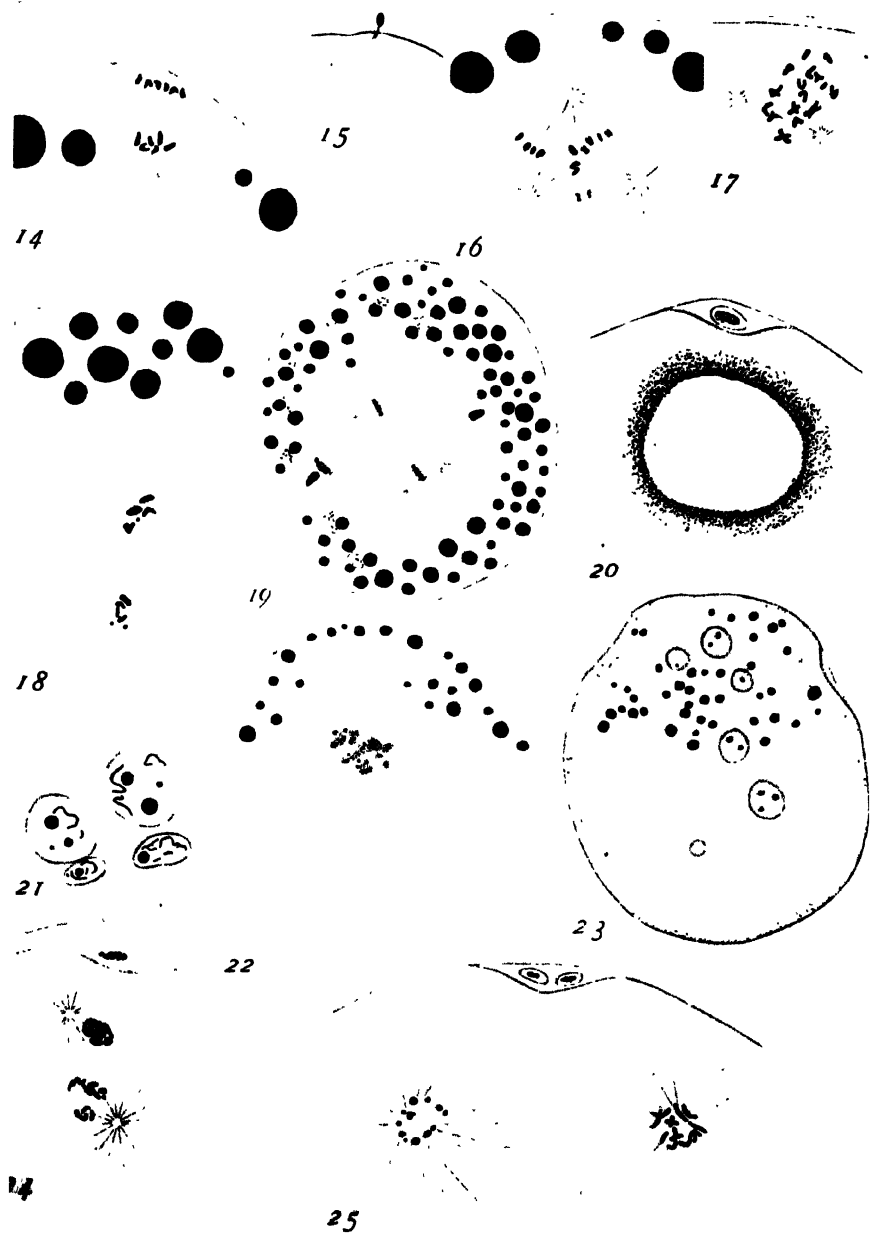


PLATE 3

EXPLANATION OF FIGURES

- 26 The same.
- 27 Each germ nucleus is dividing by itself; 115 minutes after insemination.
- 28 Disintegration of the germ nuclei; 115 minutes after insemination.
- 29 Tripolar spindle; 120 minutes after insemination.
- 30 Degeneration of the germ nuclei; 120 minutes after insemination.
- 31 to 34 Eggs radiated both before and after fertilization.
- 31 Abnormal polar body formation; 60 minutes after insemination.
- 32 The same.
- 33 The egg showing the phenomenon of budding.
- 34 The same; both 120 minutes after insemination.



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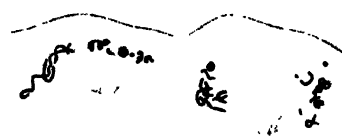
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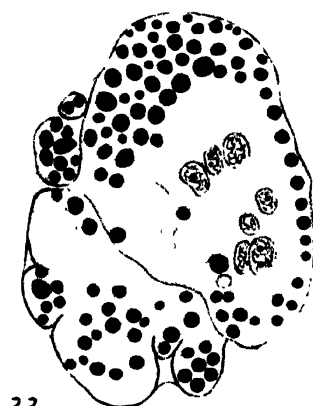
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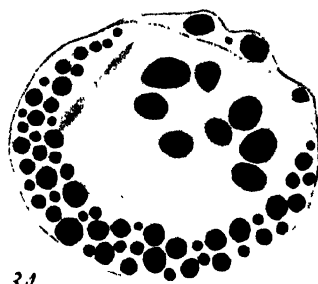


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A FURTHER STUDY OF SIZE INHERITANCE IN DUCKS WITH OBSERVATIONS ON THE SEX RATIO OF HYBRID BIRDS

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SEVEN CHARTS

In a previous number of this journal the writer reported the result of a cross between two races of ducks differing in size. The number of offspring produced was small, but tended to show a marked increase of variability in size in F_2 .

In the breeding season of 1912 more data were obtained from the same stocks, 57 F_1 and 31 F_2 ducks being raised to maturity. Growth curves were made for all these ducks from an early age until such time as they were considered adult, or nearly so. The average length of this period is 142 days. The growth curves give a good check on the adult or autumn weight of the individual, and enable one to assign the proper weight figure to each bird with very fair precision. The ducks were weighed at intervals of one week to two weeks.

The curves will be considered later on, together with the growth rate of a race of pure wild mallards reared in 1912.

As a further test of the parent stocks, which it may be recalled consisted respectively of Rouen ducks and domesticated mallards of a particular strain, 9 pure Rouen and 20 mallards were reared in 1912. The weight of these animals at five months of age is recorded in table 8.

To produce another lot of F_1 ducks, one of the original Rouen males was mated with three female mallards taken at random from among the same birds used in the previous work. For the F_2 generation, the same three F_1 females as had been used the previous year, Nos. 83, 101, and 106, were mated with F_1 male

TABLE 1
Summary of entire series of observations on weight in Rouen and mallard ducks, and in hybrids between these races

	GROUP	AGE	NUMBER OF MALES	NUMBER OF FEMALES	MEAN WEIGHT OF MALES IN GRAMS		MEAN WEIGHT OF FEMALES IN GRAMS		STANDARD DEVIATION OF MALES	STANDARD DEVIATION OF FEMALES	C. V. MALES	C. V. FEMALES
Rouen.....	original stock	1½ years	2	4	2790	2535						
	2d gen.	5 mos.	2	5	2535	2396						
	3d gen.	5 mos.	6	9	2250	2149						
	4th gen.	5 mos.	4	5	2610	2330						
	2, 3 and 4 combined	5 mos.	12	19	2417	2261	225	171	9.3	7.56		
Mallard.....	original stock		about 30	about 35								
	2d gen.	5 mos.	59	43	1068	928	119	76	11.14	8.18		
	3d gen.	5 mos.	7	13	1148	1074	112	148	9.76	13.6		
1910 and 1911 hybrids...	F ₁ gen.	5 mos.	10	3	1665	1587	97	54	5.82	3.43		
	F ₂ gen.	134 to 151 days	17	16	1781	1634	224	181	12.37	11.07		
	F ₁ gen.	134 to 160 days	36	21	1660	1479	173	154	10.42	10.5		
1912 hybrids.	F ₂ gen.	133 to 159 days	17	14	1700	1546	306	162	18.00	10.5		
	F ₁ gen.	5 mos.	46	24	1661	1492	158	152	9.5	10.1		
	F ₂ gen.	5 mos.	34	30	1740	1592	269	174	15.4	10.9		

TABLE 2
Summary of measurements of 1912 ducks in centimeters

			BILL	TARSUS	NECK	TOTAL LENGTH
Males	F ₁	mean	5.5	5.24	35.6	68.2
		S.D.	0.27	0.31	1.69	2.53
		C.V.	4.90	5.91	4.74	3.71
	F ₂	mean	5.5	5.12	35.5	68.0
		S.D.	0.29	0.29	2.22	3.67
		C.V.	5.27	5.66	6.26	5.39
Females	F ₁	mean	5.21	5.0	32.9	61.2
		S.D.	0.22	0.19	1.31	3.54
		C.V.	4.22	3.80	3.98	5.78
	F ₂	mean	5.35	5.0	32.3	63.1
		S.D.	0.24	0.19	1.98	2.51
		C.V.	4.48	3.80	6.13	3.97

103, for the first three clutches of eggs, with F₁ male 105 for the last two clutches (see former paper). Female 83 died after the first few eggs had been taken from the pen, so that nearly the entire F₂ lot was raised from only two mothers. It is thus evident that as regards the number of the parent individuals in each lot there is fully as great a chance for variability among the F₁ ducks as among the F₂ ducks. Both generations ran together in the rearing yard and in the maturing yard in exactly the same way as in 1910-1911. The weights of the birds reared in 1912 are given in tables 4 to 7. In table 1 are given the means and certain other statistical constants for each lot of birds together with the results obtained in the two previous years, thus summarizing the entire experiment.

The standard deviation in weight of the 1912 birds is greater than that of the 1910 and 1911 birds. The mean weight of the 1912 birds is somewhat less than that of the 1910-1911 birds, especially the F₂ birds.

It is plain that, in spite of the greater variability of the 1912 F₁ birds, induced no doubt mostly by their greater number (57 animals instead of 13), there is still a small excess of variability of the F₂ over that of the F₁ generation (at least among the males).

Finally, the combination of the three years' work gives a very satisfactory basis for comparison, the F₁ lot comprising 70 animals

and the F_2 lot 64. The results for 1912 are not, however, so striking as those for 1911, but point in the same direction—to a partial segregation of multiple size factors, or to a modification of the gametes brought about by their association in F_1 zygotes. The increased variability in F_2 is far greater in the male sex than in the female.

As a further index of size differences, external measurements were taken on all the adult 1912 ducks. These were as follows:

Bill: exposed culmen.

Tarsus: the tibio-tarsal measurement as used by the ornithologists.

Neck: from the anterior point of the breast bone to the tip of the bill. The front end of the breast bone is a point which can readily be felt. This measurement is easier after rigor mortis has passed.

Length: tip of bill to end of tail feathers.

A summary of these measurements is given in table 2, for each sex and generation. Among the eight pairs of coefficients of variability obtained the F_2 coefficient is greater than the F_1 coefficient in five cases, the same in one case, and less than the F_1 in two cases. Accordingly greater variability of the F_2 birds is not very clearly shown in the measurements taken.

GROWTH OF DUCKS

In a recent paper Goldschmidt gives the result of some size work in ducks. He does not deal with adult weights, but with a coefficient of growth determined by dividing the weight at ten weeks by the first or hatching weight. In this way he investigated the early growth rate of several breeds. He obtained growth figures for one lot of F_2 Peking female mated with wild mallard male, and records a great divergence, which he attributes partly to segregation. It is to be remarked that Goldschmidt's wild mallard race reached a weight of 1470 grams, which would suggest that it might have been contaminated, for this weight is very high. The average for my original stock of wild mallards is 1073 grams for the males, and 922 grams for the females, while the autumn, or five month's weights for the offspring were 949 and

892 grams respectively. The difficulty of obtaining pure wild mallards in parts of Europe is well brought out by Rogeron in his work "Les canards."

Goldschmidt lays stress on the importance of environmental factors in the growth rate of ducks. To show the influence of earliness or lateness of hatch on body weight, I have prepared table 3. In this table each of the four groups of 1912 ducks (both sexes and both generations) were split into early and late hatches, and the average age and average adult weight found for each of the eight groups thus obtained. The result is very striking, and shows the advantage of lateness over earliness in every one of the four cases.

The growth curves show the same phenomenon. For example in chart 4, curve $F_2\varphi 1$ represents the weights of early hatched birds and shows the greatest weight at 60 days of age but a very low maturity weight at 105 days. On the other hand, curve $F_2\varphi 5$ (late hatched birds) shows a smaller weight at 60 days of age but much greater weight at maturity.

The early hatched birds tend to mature quicker and remain smaller. This result only emphasizes the importance of outside conditions as pointed out by Goldschmidt.

In charts 2 to 5 growth curves are given for both sexes and both generations of the cross-bred birds. Each curve represents the average growth of one sex of each clutch of eggs. At the point marked *P*, at 50 to 70 days of age, there is always a depression which corresponds with the removal of the ducklings from the rearing yard to the maturing yard, where the free access to water and greater space tended for a short time to keep their weight down.

There is a slight tendency to a depression at the time of moult from the first juvenile to the first adult plumage, which takes place between the ages of 110 and 140 days. In all cases the birds were in full plumage when killed, and the completion of the full plumage is a very good criterion of the time when the adult or autumn weight has been attained. Increase in weight beyond this point seems to be due mostly to an increase of fat, and is generally much more marked among the females than among the

TABLE 3
Relation between size of 1912 ducks and early or late hatching

F ₁ GENERATION		F ₂ GENERATION
19 early males.....	approximate date of hatching, May 27 approximate age when killed, 140 days average weight in grams, 1532 grams approximate date of hatching, June 20	11 early hatched males..... approximate date of hatching, May 27 approximate age when killed, 140 days average weight in grams, 1609 grams approximate date of hatching, June 18
17 late males.....	approximate age when killed, 140 days average weight in grams, 1743 grams approximate date of hatching, May 29	6 late hatched males. approximate age when killed, 145 days average weight in grams, 1873 grams approximate date of hatching, May 24
11 early females...	approximate age when killed, 145 days average weight in grams, 1404 grams approximate date of hatching, June 18	7 early hatched females..... approximate age when killed, 148 days average weight in grams, 1405 grams approximate date of hatching, June 19
10 late females.....	approximate age when killed, 140 days average weight in grams, 1562 grams	7 late hatched males. approximate age when killed, 146 days average weight in grams, 1687 grams

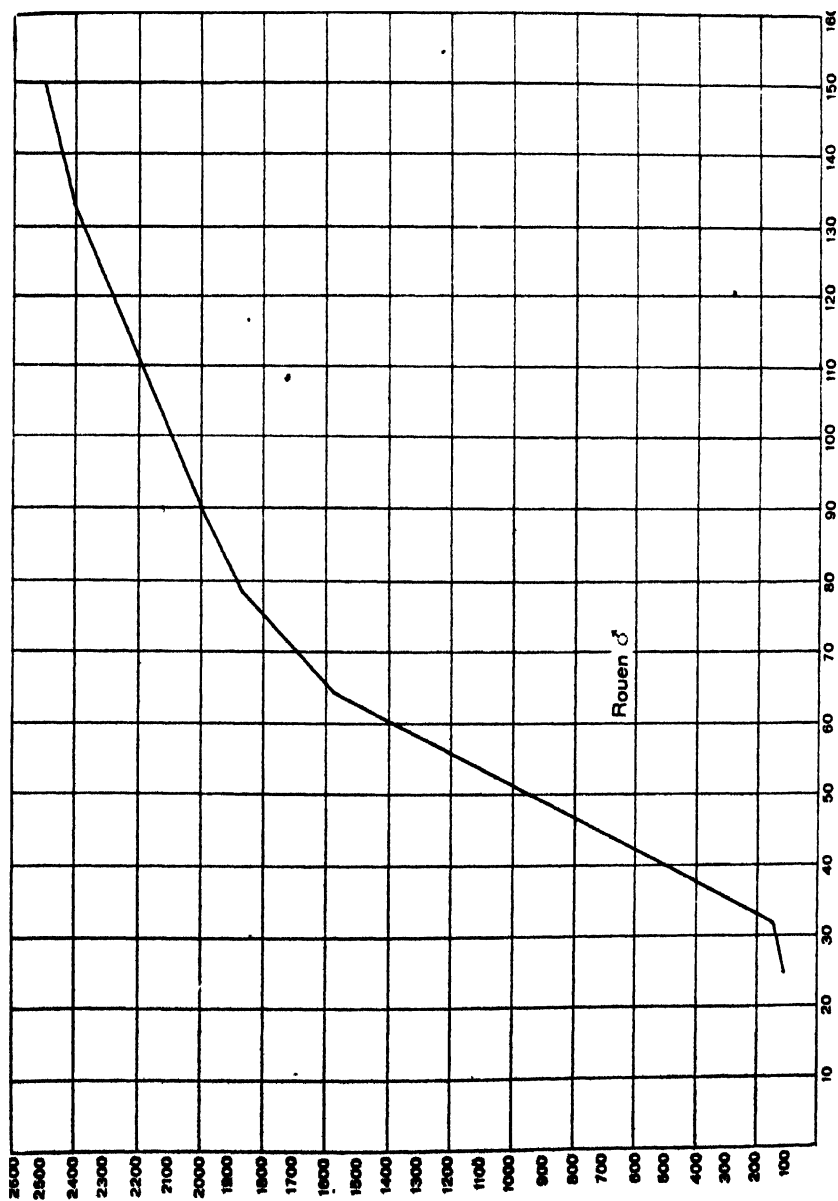


Chart 1 Growth curve of male Rouen chicks, plotted in days of age and grams of weight

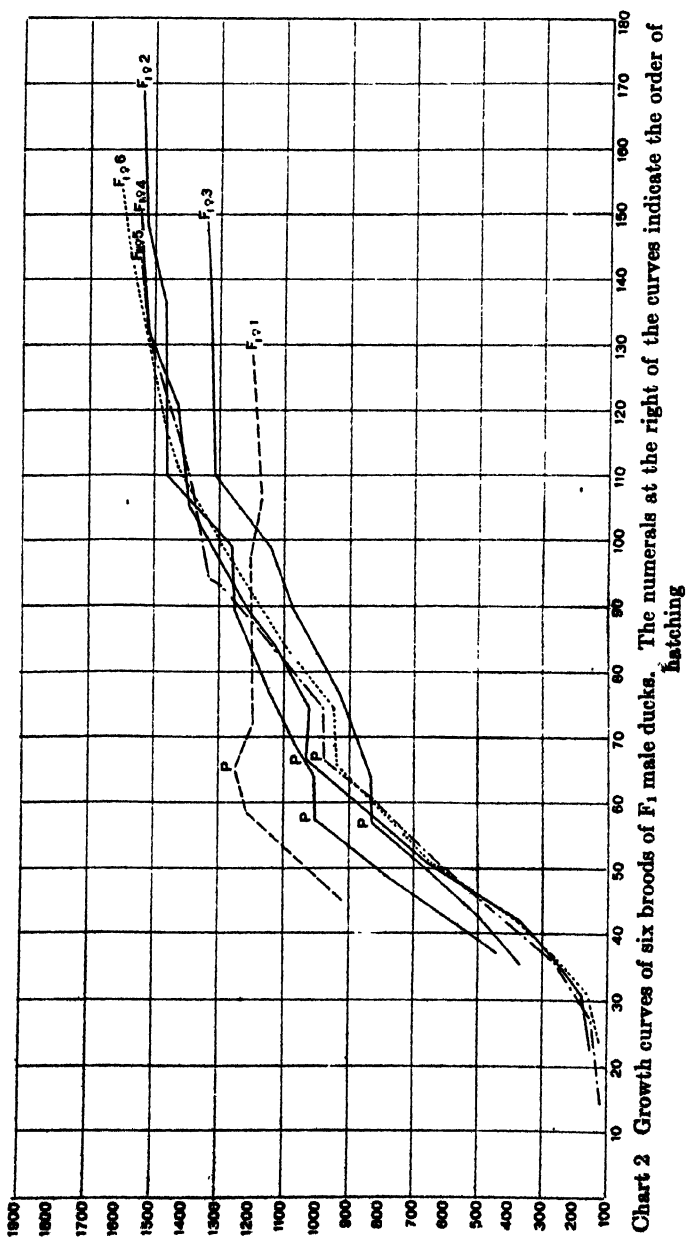


Chart 2 Growth curves of six broods of F₁ male ducks. The numerals at the right of the curves indicate the order of hatching

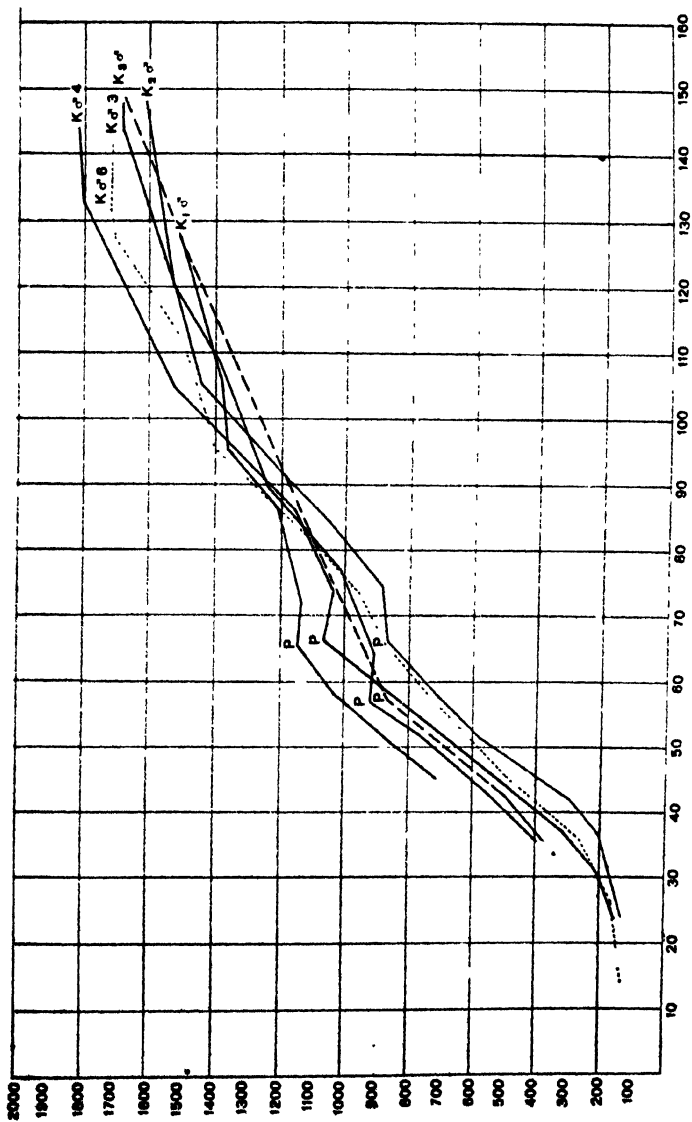
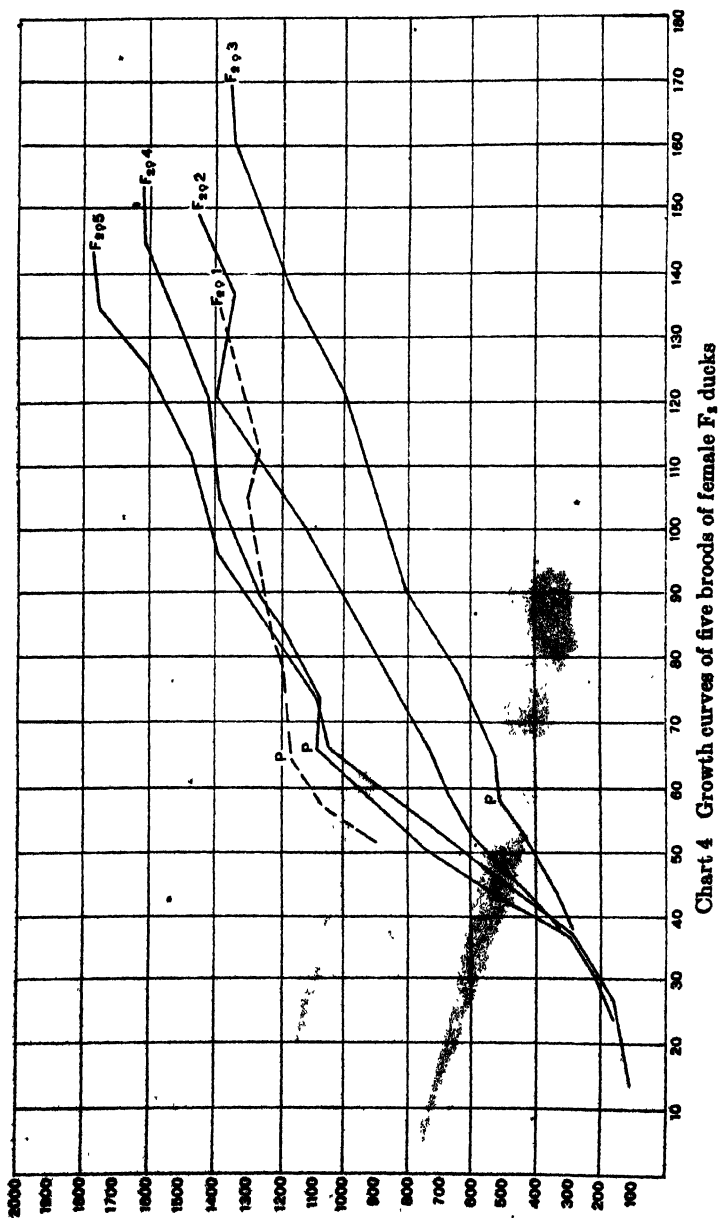


Chart 3 Growth curves of six broods of F_1 female ducks

Chart 4 Growth curves of five broods of female F_1 ducks

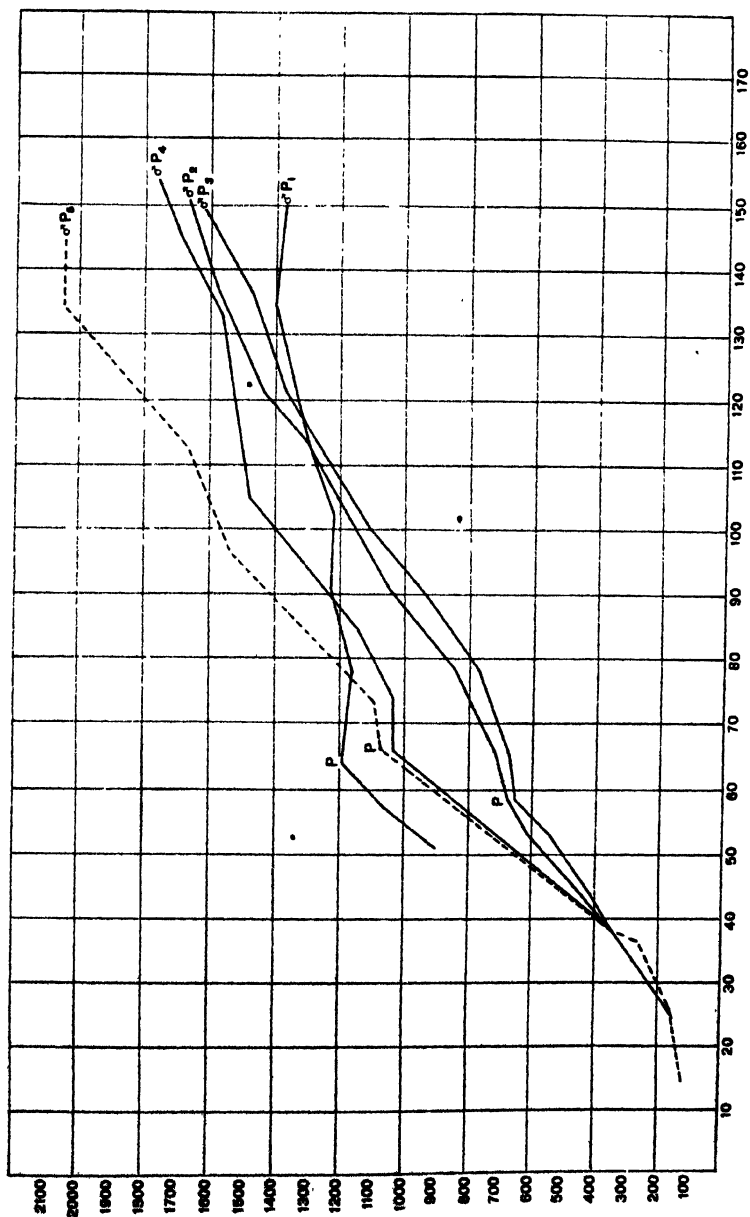


Chart 5 Growth curves of five broods of F_2 male ducks

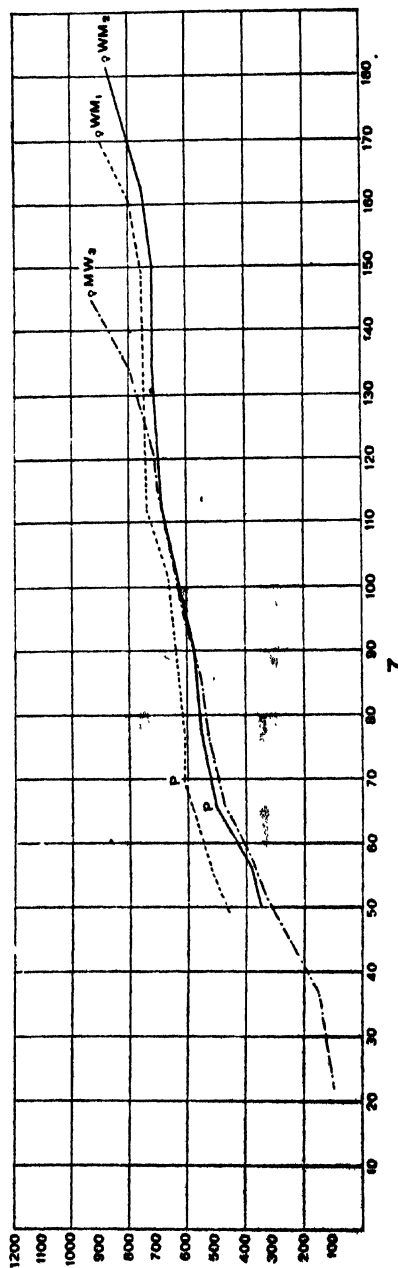
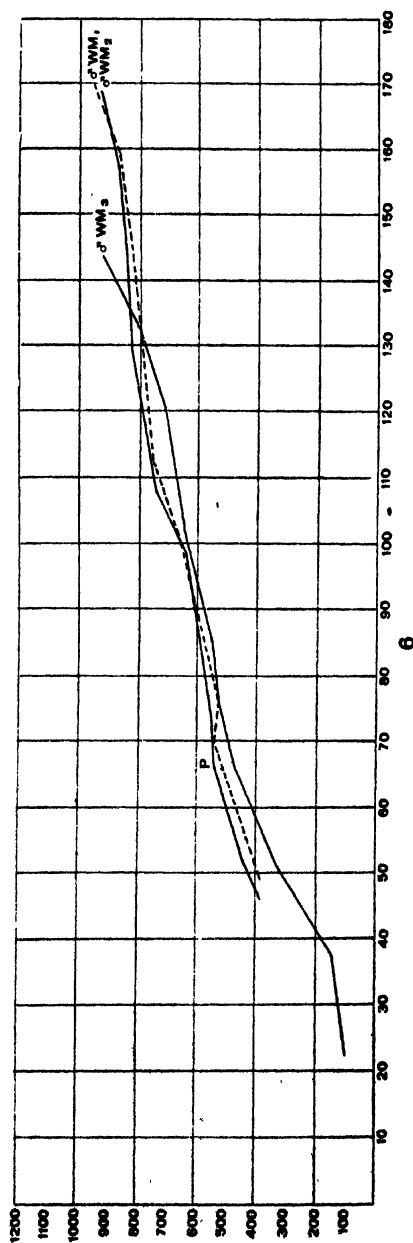


Chart 6 Growth curves of three broods of pure wild male mallards
 Chart 7 Growth curves of three broods of pure wild female mallards

males. The small figures at the end of the curves refer to the sequence in date of hatching time of the different clutches of eggs.

The curve for the Rouen ducks shows the remarkable growth of this race between the ages of 30 and 70 days. The curves for the small race are not available, but I have added two curves for 26 individuals of a pure wild race of mallards in charts 6 and 7. These birds are slightly smaller and much more uniform in size than the 'English' mallards. They show a standard deviation of 43.5 for the males and 23.93 for the females, and a coefficient of variability of 4.5 for the males and 2.6 for the females. The males, as among the other races, show a much greater tendency to vary. This fact may be a universal one for ducks; it is in agreement with the more uniform c. v. for the females in both F_1 and F_2 generations.

INHERITANCE OF COLOR IN ROUENS

This is mentioned because Goldschmidt (p. 189) expresses some doubt as to the homozygous nature of the color of the Rouens. In my strain at least there is no question about this. In crosses with mallards, 46 F_1 males were all true to the mallard type of pattern, while 34 F_2 males gave only two birds which were slightly 'off color.' These two birds had large white collars with some white on the breasts and primaries. Goodale showed in crosses between Pekins and Rouens that a very complicated assemblage of color types resulted in the first hybrids. He attributes this to heterozygosis among the Pekin males, which view would appear to be the correct one.

THE SEX RATIO OF F_1 SIZE DUCKS

Owing to the present general interest in disturbed sex ratios of hybrid animals it is worth while to call attention to the results obtained in ducks (table 1). In both seasons there was a great preponderance of males among the F_1 offspring though among the F_2 birds, where the parents were similar in size, the sexes are nearly equal. Adding the results for both years together, we obtain

the following proportions of the sexes: In F_1 , males 46, females 24; in F_2 , males 34, females 30.

In the first case is found practically a two to one ratio, which, in view of the large number of observations, is significant. Guyer called attention to an excess of males in various hybrid birds, especially when the parents were from widely separated species or different genera. His results are amply confirmed in a recent paper by Goeffrey Smith and Mrs. Haig-Thomas on pheasants. An excess of males is apparent in fertile hybrids, but seems to be greater in sterile hybrids. I myself have found that in hybrids between Reeves and Torquatus pheasants, Jungle fowl crossed with pheasants, and Swinhoe pheasants crossed with Silver, a great excess of males occurs.

It is therefore very interesting to find the same result obtaining in a cross between two domestic races of ducks, very dissimilar in size, but derived from the same wild species, and producing fertile offspring when intercrossed. It is possible to explain the inequality of the sexes as due to a selective death rate on the part of the two sexes, a larger proportion of male zygotes surviving, but the supposed selective death rate, if it occurs at all, must be effective very early in embryonic life, for out of 76 eggs set in 1912, 60 hatched, and 57 were reared to maturity, producing 36 males and 21 females. Most of the eggs, which failed to produce young were sterile or at least discarded as such after ten days incubation (exact number not noted) so that a selective fertilization seems to be a more probable explanation in this case, but an early embryonic selective mortality cannot be ruled out.

In conclusion I wish to express my thanks to Professor Castle for help in working out details.

SUMMARY

1. The high coefficient of variability of both the parent races, especially the mallards, may perhaps increase the variability in F_1 . Possibly the mallard race may contain an admixture of the small, so called 'toy' race, which would account for some of the small individuals which occasionally appear. In spite of this, however, the male ducks show a very considerably increased variability.

of weight in F_2 over their variability in F_1 and the females show a very small increase. The numbers are now thought large enough to have a significance.

2. If a stock like the pure wild race mentioned above, could be used as a small parent, it is very likely that a much more striking segregation of size might be observed.

3. The present experiment does not throw any further light on the theoretical side of the question except to diminish the possibility of the existence of any large and clear cut size units in birds, which would result in an easily recognized numerical ratio.

4. The male sex is much more variable in size than the female.

5. The Rouen race used in this work was homozygous for color (mallard color) when crossed with a mallard race.

6. The growth curves show that a very satisfactory age for studying size of ducks is 140 to 150 days, at the assumption of the first adult plumage, but also point to a marked effect of earliness and lateness of hatch on the rate of growth and ultimate size. This difference probably varies greatly from year to year and cannot be regarded as uniform.

7. A disturbed sex ratio occurs among the F_1 hybrids, a result apparently of the difference in size of the parents, for it is not seen in F_2 . This ratio has resulted in the preponderance of males over females in the proportions of nearly two to one.

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TABLE 4

Weights and measurements of F₁ male ducks reared in 1912

NO.	DATE OF HATCHING	DATE OF KILLING	WEIGHT	BILL	TARSUS	NECK	TOTAL LENGTH
			grams	cm.	cm.	cm.	cm.
305	May 23	Sept. 28	1740	5.9	5.5	35.5	68.0
306	May 23	Sept. 28	1290	5.3	5.1	31.5	61.2
307	May 23	Sept. 28	1470	5.6	5.3	35.5	67.3
308	May 23	Sept. 28	1830	5.8	5.5	34.3	67.1
310	May 23	Sept. 28	1100	4.9	4.6	28.7	58.5
312	May 23	Sept. 26	1760	6.1	6.5	38.5	72.0
313	May 30	Nov. 6	1690	5.9	4.5	37.0	70.5
314	May 30	Nov. 6	1700	5.5	5.3	37.1	69.9
318	May 30	Nov. 6	1640	5.6	5.3	36.0	69.8
319	May 30	Oct. 14	1600	5.3	5.0	35.8	68.2
320	May 30	Oct. 26	1720	5.4	5.4	36.3	69.0
321	May 30	Oct. 26	1500	5.9	5.4	36.0	66.6
323	May 30	Oct. 26	1590	5.6	5.3	35.0	67.0
325	May 30	Oct. 14	1500	5.3	5.15	35.0	66.5
326	May 30	Oct. 14	1600	5.5	5.15	36.8	69.0
344	May 30	Oct. 14	1600	5.8	5.2	36.8	70.0
346	May 30	Oct. 26	1460	5.2	4.9	35.7	66.8
347	May 30	Nov. 6	1770	5.4	5.1	35.5	68.5
348	May 30	Nov. 6	1550	5.2	5.1	35.1	66.2
351	May 30	Nov. 6	1680	5.4	5.3	36.2	69.0
354	June 15	Oct. 26	1655	5.3	5.3	36.1	69.2
358	June 15	Nov. 6	1860	6.1	5.5	35.0	69.0
360	June 15	Nov. 6	1890	5.8	5.2	37.2	70.4
361	June 15	Oct. 26	1570	5.4	5.3	37.6	70.5
365	June 15	Oct. 26	2130	5.6	5.3	36.5	70.8
370	June 15	Nov. 15	1700	5.8	5.4	33.6	66.8
371	June 15	Nov. 6	1560	5.4	5.0	37.8	69.7
373	June 15	Nov. 6	1760	5.4	5.2	35.0	68.6
375	June 15	Nov. 15	1700	5.4	5.2	35.5	68.6
378	June 25	Nov. 15	1750	5.5	5.2	34.5	68.3
379	June 25	Nov. 15	1630	5.9	5.4	36.2	69.8
381	June 25	Nov. 15	1800	5.7	5.1	35.4	68.8
383	June 25	Oct. 14	1700	5.2	5.7	35.5	67.0
385	June 25	Nov. 15	1800	5.8	5.5	36.2	69.9
387	June 25	Nov. 6	1700	5.3	5.1	35.0	67.5
390	June 25	Nov. 6	1770	5.9	5.2	36.4	69.7

TABLE 5

Weights and measurements of F₁ female ducks reared in 1912.

NO.	DATE OF HATCHING	DATE OF KILLING	WEIGHT	BILL	TARSUS	NECK	TOTAL LENGTH
			grams	cm.	cm.	cm.	cm.
309	May 23	Sept. 28	1200	5.4	5.0	33.0	62.9
315	May 30	Oct. 14	1500	5.1	5.0	31.6	64.0
317	May 30	Oct. 26	1560	5.1	5.2	33.8	65.0
324	May 30	Oct. 26	1385	5.3	5.1	32.2	63.9
327	May 30	Nov. 6	1580	5.2	4.9	32.3	63.4
330	May 30	Nov. 6	1420	5.6	5.0	34.2	66.0
335	May 30	Nov. 26	1450	5.3	4.8	33.1	63.8
338	May 30	Nov. 26	1230	4.9	4.7	31.5	61.8
340	May 30	Oct. 14	1500	5.15	4.9	34.0	62.0
342	May 30	Oct. 14	1330	5.0	4.8	32.0	63.0
343	May 30	Oct. 14	1290	4.8	4.7	32.1	63.0
350	June 15	Oct. 14	1390	5.1	4.9	32.4	63.6
359	June 15	Oct. 15	1700	5.6	5.4	33.5	66.0
364	June 15	Oct. 15	1600	5.3	5.1	32.5	63.8
367	June 15	Oct. 26	1530	4.9	4.9	30.8	62.3
369	June 15	Oct. 26	1380	5.3	5.0	34.2	64.4
372	June 15	Nov. 6	1900	5.7	5.5	37.2	70.7
374	June 15	Nov. 6	1540	5.2	5.1	32.5	64.0
386	June 25	Nov. 6	1450	5.1	5.0	33.6	65.0
388	June 25	Oct. 26	1610	5.3	5.2	32.7	65.7
391	June 25	Nov. 15	1520	5.1	5.1	32.2	64.8

TABLE 6

Weights and measurements of F₂ male ducks reared in 1912

NO.	DATE OF HATCHING	DATE OF KILLING	WEIGHT	BILL	TARSUS	NECK	TOTAL LENGTH
			grams	cm.	cm.	cm.	cm.
247	May 17	Oct. 14	1330	5.25	4.7	35.0	66.0
288	May 17	Sept. 28	1360	5.85	5.1	35.5	66.3
311	May 30	Oct. 26	2180	5.6	5.0	38.0	72.3
316	May 30	Oct. 13	1700	5.65	5.4	37.0	69.0
328	May 30	Oct. 26	1680	5.4	5.2	38.0	69.8
329	May 30	Oct. 13	1490	5.1	5.1	34.6	65.0
331	May 30	Nov. 6	1645	5.5	5.2	36.0	69.0
332	May 30	Nov. 15	1945	6.0	5.6	38.0	72.0
336	May 30	Oct. 13	1670	5.5	4.9	36.0	67.0
341	May 30	Oct. 26	1100	4.8	4.5	29.7	58.4
349	May 30	Nov. 15	1600	5.8	4.5	36.7	69.5
352	June 15	Nov. 15	1480	5.2	5.2	33.0	65.0
356	June 15	Nov. 6	1870	5.5	5.5	35.5	69.0
362	June 15	Oct. 26	1890	5.4	5.1	35.3	69.2
368	June 15	Nov. 15	1850	5.2	5.2	32.5	66.5
377	June 25	Nov. 15	1750	5.6	5.3	34.8	68.0
399	June 25	Nov. 15	2400	5.8	5.7	38.0	75.0

TABLE 7

Weights and measurements of F₂ female ducks reared in 1918

NO.	DATE OF HATCHING	DATE OF KILLING	WEIGHT	BILL	TARSUS	NECK	TOTAL LENGTH
			grams	cm.	cm.	cm.	cm.
283	May 17	Sept. 28	1330	4.7	4.9	29.4	58.0
289	May 17	Sept. 28	1390	5.1	4.7	29.6	59.5
296	May 17	Sept. 28	1450	5.25	5.1	32.0	62.0
333	May 30	Oct. 26	1520	5.7	4.9	34.4	66.0
337	May 30	Nov. 15	1430	5.5	5.2	34.0	65.0
339	May 30	Oct. 26	1415	5.6	5.0	32.5	64.0
345	May 30	Nov. 15	1300	5.1	4.8	30.0	60.0
353	June 15	Nov. 15	1600	5.4	4.9	30.8	62.5
357	June 15	Oct. 26	1600	5.8	5.1	34.2	66.4
363	June 15	Nov. 6	1600	5.3	4.9	31.0	63.8
366	June 15	Oct. 15	1640	5.4	5.3	31.2	65.0
376	June 25	Oct. 15	1800	5.4	5.3	30.0	62.5
380	June 25	Oct. 15	1770	5.0	5.0	29.9	62.8
382	June 25	Oct. 15	1800	5.6	5.4	33.8	66.5

TABLE 8

Weights in grams at five months of age of Rouen and mallard ducks reared in 1918

ROUEN MALES	ROUEN FEMALES	MALLARD MALES	MALLARD FEMALES
2440	2290	960	830
2570	2320	1060	940
2680	2340	1120	970
2750	2340	1150	980
	2360	1150	1020
		1290	1045
		1300	1060
			1100
			1110
			1160
			1200
			1240
			1310

EXPERIMENTAL EVIDENCE CONCERNING THE DETERMINATION OF POSTURE OF THE MEMBRANOUS LABYRINTH IN AMPHIBIAN EMBRYOS

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THIRTY-EIGHT FIGURES

The purpose of the present paper is to report a series of experiments that substantiate the conclusion that the posture of the membranous labyrinth and the position of its canals is determined by some force or influence that interacts between the labyrinth and its environment. The exact nature of this control or influence has not yet been determined, but from the experiments that are to be described it is evident that it possesses a force capable of producing a complete rotation of an embryonic vesicle that has been displaced in a reversed position.

The existence of a postural influence of this kind introduces a new factor in organogenesis. It means that organs do not develop inertly in the position that they happen to find themselves: on the contrary, there is a certain amount of adjustment of position through forces interacting between them. According to the conception of what we might call the *theory of passive organ development*, all organs develop passively in the position in which they are located at the outset. Perfect form according to this theory is eventually obtained because originally the anlagen of the various organs are so perfectly placed, and all the stresses so carefully calculated, that the subsequent increase in size, and mutual pressure against each other, produce a final normal disposition of all of them. In contrast to this we may now speak of *self-placement of organs* or *individuality of organ development*, according to

which there is, in addition to the passive pressures of adjacent structures, a motive element, the *force of placement*, which helps to maintain, and corrects if necessary, the relative position of an individual organ. This *force of placement* may be defined as the product of the combined motive forces interacting between an organ and its environment. We are already familiar with the striking phenomenon of muscle migration and the movement of nerve-ganglion masses. We can now add to this the potential movement and adjustment of entire organs during their course of development, and it is not necessary to add that this *autostatic tendency* is doubtless much more pronounced in some organs than in others.

The writer's attention was first directed to the possibility of experimentally altering the position of the developing labyrinth, during some investigations on equilibration in amphibian larvae (Streeter '06). In these experiments it was found that the cells constituting the ear vesicle are specialised very early, and though transplanted to an abnormal environment they continue to differentiate themselves in the usual way into a recognizable labyrinth. Lewis ('07) showed in a subsequent paper that this was true while they were still in the stage of an uninvaginated plate, quite in contrast to the surrounding cells forming the cartilaginous capsule, which Lewis clearly proved were not predetermined in this way.

In a later paper (Streeter '07) additional evidence was given of the high degree of developmental independence possessed by the early labyrinth cells. It was shown that even fragments of the vesicle may develop independently of the rest of the vesicle, and any individual part, for example the endolymphatic appendage, may be quite normal in cases where the remainder of the labyrinth is very abnormal. It was also shown (Streeter '09) that when two primitive vesicles are crowded together into the same pocket they do not fuse and form together one large labyrinth, but remain as two distinct labyrinths. Moreover, it was shown that this developmental independence of the vesicles extends to the difference existing between a right and left-sided organ. The dextral or sinistral character, or laterality of the ear

vesicle, is not controlled by the environment but is determined by the intrinsic character of its own constituent cells. A left-sided ear vesicle when transplanted to the right side develops into a labyrinth having all the characteristics of a left-sided organ; the anterior canal is formed on the caudal border of the labyrinth, the posterior canal on the oral border, and the lagena, which normally is directed caudalward, is found extending forward toward the eye.

It was found, however, that the ear vesicle, though capable of this marked power of self-differentiation, apparently was not in all respects independent of the surrounding structures. The posture of the developed labyrinth, the situation of its canals and various chambers, seemed to be controlled by the environment. Deliberate rotation of the ear vesicle into abnormal positions and even transplantation to the opposite side of the body, always resulted in a labyrinth possessing a normal attitude with reference to the brain, ganglion masses, and the surface of the body. In all of seventeen experiments performed for this purpose the results were positive, and the writer consequently came to the conclusion that there is some influence interacting between the ear vesicle and its environment that constitutes the chief factor in the determination of its placement.

At the same time an experienced foreign investigator had been working independently upon a similar problem (Spemann '06 a and '06 b). His experiments consisted in removing the ear vesicle in young *Rana esculenta* larvae and then replacing it in an inverted position for the purpose of studying the consequent abnormal body movements and their correlation with the anatomical results of the operation. He also studied the influence of the epithelial labyrinth upon the surrounding connective tissue, and its relation to the formation of the cartilaginous and bony labyrinth. Concerning this latter problem he did not come to a definite conclusion. The former problem, however, gave positive results. He found that the inverted vesicle continued in its development and formed a more or less complete labyrinth, but, unlike my specimens, the orientation of the developed labyrinth corresponded to the operative displacement of the vesicle. There had been no subsequent readjustment of position. After the appearance of

my papers (Streeter '06 and '07) in which I reported the experiments in which, after various displacements, the labyrinths all recovered their normal postural relations, Spemann ('10) made further experiments and reported more at length with regard to postural development. His results confirmed his earlier observations, and his paper contained illustrations showing sections of larvae of different ages in which the inverted ear vesicle has remained inverted though otherwise practically normal. Out of twelve operations nine labyrinths remained inverted, two recovered partly, and one recovered perfectly the normal position. Regarding the latter specimen he surmised that it had not been sufficiently turned, and had slipped back into its normal position. The results, therefore, as obtained by Spemann are directly contradictory to those obtained by me. On account of the great embryological significance of the question of environmental postural control, further experiments were plainly warranted both for the purpose of testing my own previous results and if possible for finding some explanation of the discrepancy existing between the observations of Spemann and myself. From the study of these new experiments and from an examination of the accompanying photographs it will be plainly apparent that they confirm my previous results. Before entering into a further discussion of them we may proceed with a description of the details of the experiments themselves.

METHOD OF OPERATION

Obviously the conditions of the operation should be planned so that the environmental control would be put to an extreme test. In most of my previous experiments, and in all of Spemann's, the vesicle operated upon, after being taken out and rotated one way or another, was replaced in its own pocket. Recovery of the original posture after such an operation might be explained by the possibility of the vesicle not having been completely detached; some small strand of tissue might suffice to draw it back into the original position. Also it is conceivable that, since the vesicle and any adherent mesodermal fragment exactly fitted the pocket

from which they were taken, they might on that account slip back into their original position. Both of these objections were avoided by transplanting a vesicle from one specimen into the emptied ear pocket of another specimen. This certainly involved complete detachment, and it is obvious that the transplanted ear vesicle would not exactly fit in a pocket in which it did not originally belong. All of the following experiments were done in that way.

The experiments were carried out on larvae of *Rana pipiens*, and the operating stage was the same as that used in previous experiments (Streeter '06, fig. 3, p. 547). This is just at the end of the non-motile stage, and the outer form of the larvae shows a distinct tail bud, and the eminences on the head caused by the optic cup and head ganglia. The ear consists of an invaginated saucer-shaped mass of cells just in the process of being pinched off from the deeper layer of the skin, with the edges inverted and in the process of completing the closure of the vesicle: that is, transition from auditory cup to auditory vesicle. In this, as in previous papers, the attempt is not made to distinguish between these two stages, and the term 'ear vesicle' is used to cover both. The technique of the operation is as follows: Two larvae are removed from their gelatinous capsules and placed side by side in distilled water, under a binocular microscope. With two no. 12 embroidery needles, a linear tranverse incision is made through the ectoderm of one of the specimens over the site of the ear vesicle, the incision being about three times as long as the vesicle, and the right side of the animal always being used for the operation. The lips of the wound are then gently everted, forward and backward respectively, which discloses the thin lateral wall of the vesicle, or if this is torn in the removal of the skin then one sees the pigmented concave interior of the vesicle. With the needles the vesicle is now loosened from its pocket and cast away. This leaves an empty pocket, free for the transplantation. The right vesicle of the second specimen is uncovered in a similar way, loosened from its pocket and then slipped into the empty right auditory pocket of the first specimen. In tucking in the vesicle under the edges of the wound care was taken in all cases to place the vesicle so that the lateral or concave open surface of

the vesicle should lie against the brain, and the median or convex surface toward the opening of the wound. The lateral and median surfaces of the vesicle are easily recognized and therefore this transplantation in a reversed posture can be done with great accuracy. This method of placing the vesicle has a double advantage. It furnishes a complete rotation of 180 degrees and it is a secure way to transplant the vesicle which is very easily wedged in the pocket in that posture. The vesicle shows no tendency to escape, and as the lips of the wound are not very wide apart, it is not necessary to take any precautions about holding them together. They take care of themselves and need no superimposed weights or further attention. The specimen is now set aside and in the course of three or four hours all traces of the wound have disappeared. The specimens are allowed to go on with their development for fourteen days, the period usually necessary for the formation of the canals, at the end of which time they are preserved in a chrome-acetic mixture and are ready for examination. In most of the operations two specimens were utilized, one always being discarded after transplanting its right vesicle. In four experiments the writer succeeded in making a complete exchange of the right vesicle between the two specimens and preserving both of them. This involves great care and the additional effort required does not justify the procedure. The preserved specimens were imbedded in paraffin, cut in series and stained with hematoxylin and congo-red. Examination of them showed that nineteen of the experiments were successful, in that the transplanted vesicle had continued in its development far enough to recognize its different parts and their posture. Wax-plate models after the Born method were made of them all, and drawings of these, together with a photograph of a selected section from each series, are shown in figures 1 to 38. I may here state that these photographs were made with the apparatus belonging to my colleague, Professor Novy, and it gives me pleasure to take advantage of this opportunity to acknowledge his courtesy and his assistance in their preparation.

Other operations were performed with a view to the determination of character and degree of the environmental control, but these

will not be reported on in this paper; we limit ourselves to the question of whether environmental control does or does not exist. It may be pointed out that the above operative procedure offers a complete and severe test for the existence of such control. It is so arranged that we have for the experiment a transplanted foreign ear vesicle, which does not naturally fit in its new pocket and which is intentionally placed in a posture as abnormal as possible.

RESULTS OF THE EXPERIMENTS

At the outset it may be stated that in all cases where the labyrinth had developed into a structure with sufficient completeness for the identification of its relations, it was found that the position of its canals and various chambers as regards the surrounding structures was practically normal, in spite of the manipulation it had undergone at the time of the operation. As has previously been shown (Streeter '07, p. 433) the posture of the labyrinth can be determined both by the histological structure of its walls and by its outer form, as determined by wax-plate reproductions. The description of these morphological characteristics will not be repeated here; it will be sufficient to state that they are so definite that the various parts of the labyrinth can be recognized without difficulty, even though they happen to be incomplete or unequally developed.

For the sake of compactness table 1 is annexed in which are detailed the separate features of each of the nineteen transplanted labyrinths as found on examination. Each feature is marked 'normal', 'imperfect,' or 'absent,' indicated by the signs *N*, *I* and *O*. These are used in a liberal sense so, that 'normal' signifies practically normal, and includes structures abnormally large or small; 'imperfect' signifies quite abnormal, the characteristics, however, being sufficiently well defined for identification of the structure; and 'absent' means either completely absent or unrecognizable.

The features as listed will for the most part explain themselves. It may be mentioned, however, that by canal planes is meant the relative position of the canals as regards each other. Planes

drawn through the anterior and posterior canals would meet at an angle of about 95 degrees at the crus commune. This is quite constant in all of the labyrinths. The lateral canal is about perpendicular to the other two. At the end of two weeks the planes are not so well defined as they are later, but when the canals are otherwise normal the planes can be determined, even as early as that.

From a study of this table it will be seen that we can arrange our experiments in three groups, based on the completeness of the

TABLE I

LABYRINTH NO.	POSTURE	SIZE	ANTERIOR CANAL	POSTERIOR CANAL	LATERAL CANAL	CANAL PLANES	CRUS COMMUNE	VESTIBULE	LAGENA	ENDOLYMPHATIC APPENDAGE	ACOUSTIC NERVE	ACOUSTIC GANGLION	CARTILAGINOUS CAPSULE
<i>Normal group</i>													
I	N	N	N	N	N	N	N	N	N	N	N	N	N
II	N	N	N	N	N	N	N	N	N	N	N	N	N
III	N	N	N	N	N	N	N	N	N	N	N	N	I
IV	N	N	N	N	N	N	I	N	N	O	N	N	N
V	N	N	N	N	N	N	N	N	N	N	N	N	I
VI	N	N	N	N	N	N	N	N	N	N	N	N	N
VII	N	N	N	N	I	N	N	N	O	N	N	N	N
VIII	N	N	N	I	N	N	I	N	N	N	N	N	N
<i>Abnormal group</i>													
IX	N	N	N	N	I	N	N	I	O	N	N	N	N
X	N	N	I	N	N	N	N	N	O	O	N	N	N
XI	N	N	N	N	I	N	N	N	O	N	I	N	N
XII	N	N	N	N	I	N	N	I	O	N	N	N	N
XIII	N	I	N	I	N	N	N	N	O	O	N	N	N
XIV	N	I	I	I	N	N	I	I	O	O	N	N	N
XV	I	N	I	N	I	N	I	N	O	N	N	N	N
XVI	N	N	I	I	N	N	I	N	N	N	I	N	N
<i>Doubtful group</i>													
XVII	N	N	N	I	I	N	I	I	O	O	I	I	N
XVIII	N	N	I	I	N	I	I	I	?	O	I	N	N
XIX	?	I	O	O	?	?	?	?	N	O	O	O	N

resultant development of the transplanted labyrinth. These are: firstly, those in which the labyrinth is nearly normal; secondly, those in which the labyrinth is abnormal though the main parts of it are easily recognizable; and thirdly, those in which there might be some question as to the identification of the different parts of the labyrinth. There are eight in the first group, eight in the second group, and three in the last group. Based on their order in these groups we may now proceed to the examination of the individual labyrinths.

Labyrinth I: (figures 1 and 2). The photograph shown in figure 1 is taken from Series X 2 C', slide 5, row 3, section 7. The study of this labyrinth shows only one slight departure from the normal, and that concerns the Sacculus endolymphaticus which lies somewhat cephalad to the crus commune. It is on this account that it does not show in figure 1, which is directly through the crus. What looks as though it might be the edge of the sac, is the pigment layer of the skin. Aside from its slight anterior displacement it bears the usual relation to the chorioidal roof of the fourth ventricle and is normal in size.

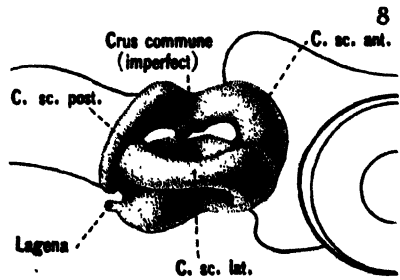
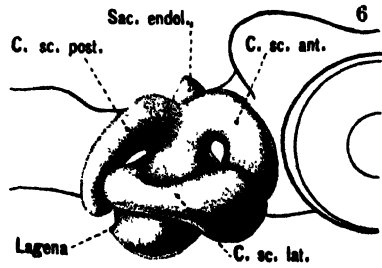
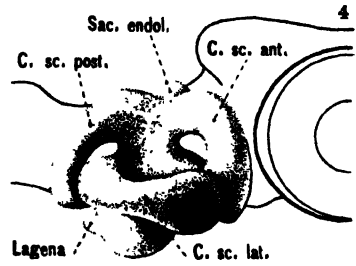
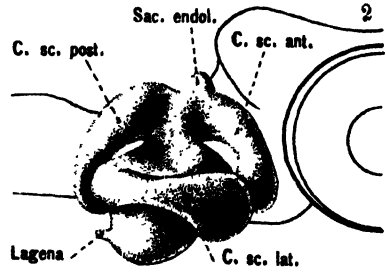
Labyrinth II: (figures 3 and 4). Figure 3 is taken from Series X 2 A, slide 4, row 1, section 3. Here as in Labyrinth I the endolymphatic sac is slightly in front of the crus commune. The crus is a little wider than in the former, so both it and the sac can be seen in figure 3. This labyrinth shows no noteworthy departure from the normal in either its histology or general form, aside from the slight displacement of the endolymphatic sac. In the drawing of figure 4 the groove between the ampullae of the anterior and lateral canals has been exaggerated a little more than is warranted by the reconstruction. In figure 3 the thickening of the ventro-lateral labyrinth wall is partly due to the crista of the lateral canal which it represents, and partly is due to the obliquity of the section of the ampulla. The characteristic relation of the endolymphatic sac to the chorioidal roof of the ventricle can be seen. The large ganglion on the ventro-median wall of the labyrinth is the proötic ganglion; the acoustic ganglion lies in a similar position but is found in the more caudal sections. Some of

the acoustic root fibers, however, can be seen entering the side wall of the brain near the endolymphatic duct.

Labyrinth III: (figures 5 and 6). Figure 5 is taken from Series X 7 A, slide 4, row 2, section 4. In the reconstruction of this labyrinth there is apparently nothing abnormal. And on examination of the section the only departure from the normal is a slight deficiency in the cartilaginous capsule in the region of the lateral center. The parabasal plate seems normal. The ganglion shown in figure 5 is the proötic near its junction with the acoustic ganglion. The section passes through the endolymphatic sac, the crus commune and the lateral semicircular canal. It shows the intimate relation existing between the endolymphatic sac with the chorioidal membrane.

Labyrinth IV: (figures 7 and 8). Figure 7 is taken from Series X 11 B, slide 3, row 1, section 5. This section was selected because it shows the characteristic protrusion of the lagena on the ventromedian wall. The section is toward the caudal end of the labyrinth and passes through, besides the lagena and large vestibule, the posterior canal and the bulging caudal edge of the lateral canal. Sections a little in front of this show the lateral canal opening into the vestibule. Evidently in the process of transplanting this vesicle some injury was inflicted on the cells that were to form the middle part of the median surface. The resultant defect includes the absence of the endolymphatic appendage and a marked imperfection in the crus commune. Otherwise the general form of the labyrinth is quite perfect, as can be seen in figure 8.

Labyrinth V: (figures 9 and 10). Figure 9 is taken from Series X 1 B, slide 2, row 3, section 5. The section passes through the endolymphatic sac, the short crus commune, and the lateral canal. The lateral vestibular wall is thin and distended and represents the dropsical type, which is a common deformity. There is a large acoustic ganglion connected centrally with the brain. This section shows well the relation between the endolymphatic sac and the chorioidal membrane. In figure 10 the shading of the lateral vestibular wall makes it look thinner than shown in the model and does not give the swollen appearance it



ought to have. The lateral wall in reality presses directly against the lateral canal. Aside from this dropsical tendency the general form of the labyrinth is quite normal. There is some deficiency in the cartilaginous capsule, particularly in the lateral center.

Labyrinth VI: (figures 11 and 12). Figure 11 is taken from Series X 4 A, slide 7, row 1, section 4. This section passes through the endolymphatic sac, crus commune, vestibule with its thickened macula acustica, and the lateral canal. The endolymphatic sac comes in contact with the chorioidal membrane in sections oral to this. Both histologically and in its general form this labyrinth is quite perfect. Figure 11 is the only photograph in the series that was retouched in any way. Here the negative was scratched to show the outlines of the lateral canal and lateral wall of the vestibule more distinctly.

Labyrinth VII: (figures 13 and 14). Figure 13 is taken from Series X 7 B, slide 6, row 3, section 3. The section passes through the endolymphatic sac, the crus commune, and the combined vestibule and lateral canal. The lateral canal exists only as a lateral pouch from the general vestibular cavity. A partial separation is indicated by an indentation on its dorsal surface, as can be seen in figure 14. The indentation does not completely perforate the pouch, so the canal is incomplete. There seems to be a general defect of the caudal portion of the labyrinth. So that, in addition to the imperfect lateral canal, the lagena is absent and the posterior canal small though otherwise complete. The acoustic ganglion is large (figure 13) and has a well developed root connecting it with the brain. The endolymphatic sac presents the usual relation to the chorioidal membrane.

Labyrinth VIII: (figures 15 and 16). The photograph shown in figure 15 is taken from Series X 2 B, slide 5, row 1, section 2. In the process of embedding and mounting this series, some of the sections were injured, though not enough to interfere with the identification of the different parts. Thus it can be plainly seen that figure 15 passes through the endolymphatic appendage, the crus commune, the vestibular pouch, and the lateral canal. The anterior half of the labyrinth is quite perfect, as can be seen in

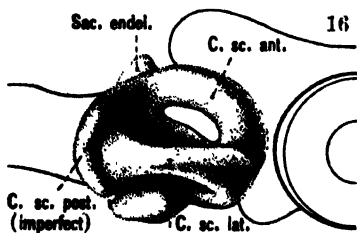
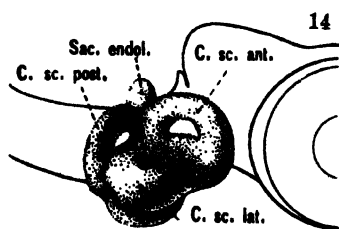
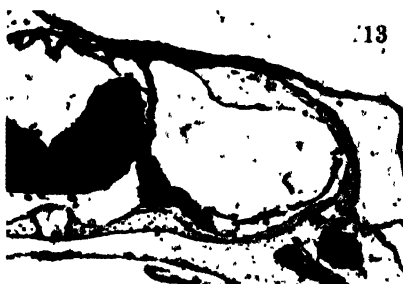
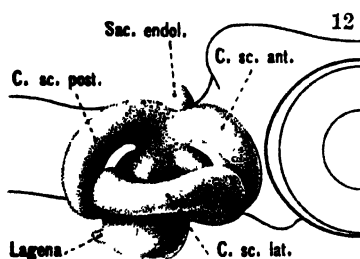
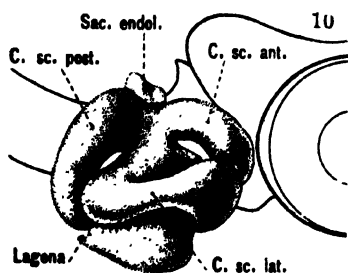
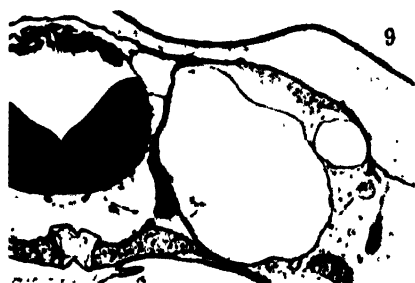
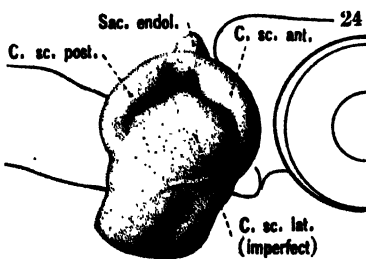
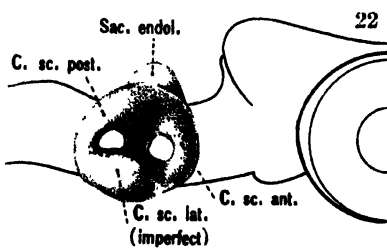
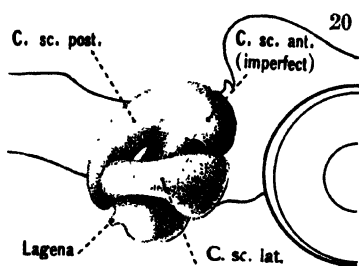
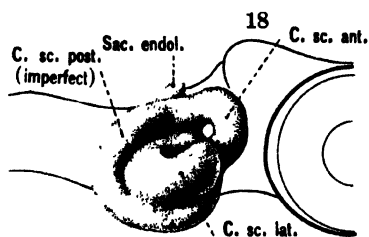


figure 16. The posterior region is defective in that the posterior canal and crus commune consist of a common pouch, taking however the usual shape of the posterior canal. The lagena developed as a pair of short pockets, one extending medially and the other caudally. Apparently the anlage was divided. The endolymphatic sac (figure 15) presents the usual relation to the chorioidal membrane.

Labyrinth IX: (figures 17 and 18). The photograph shown in figure 17 is taken from Series X 8 A, slide 2, row 4, section 9. It passes through the endolymphatic sac, the crus commune, and the vestibule with the lateral canal opening into it. In the sections oral to this the lateral canal becomes completely separated, though it is small and is deficient in the region of the ampulla. The anterior canal is small but well formed. The posterior canal is completely formed but lies closely against the vestibular wall. The vestibule is small throughout and lacks the lagena. The acoustic ganglion is well developed and is connected with the brain in the normal way. The endolymphatic sac (fig. 17) bears the usual relation to the chorioidal membrane. This is the first of what we have grouped as abnormal labyrinths.

Labyrinth X: (figures 19 and 20). The photograph shown in figure 19 is taken from Series X 11 C, slide 1, row 2, section 8. In this labyrinth the injury involves the endolymphatic appendage and the anterior canal. The latter exists as a blind pouch extending orally from the crus commune. This should be compared with figures 26 and 28, where a similar defect involves the posterior canal. The remainder of the labyrinth is quite perfect. Figure 19 shows the crus commune, the vestibule, with a portion of the acoustic ganglion, and the lateral canal. The endolymphatic appendage is entirely absent.

Labyrinth XI: (figures 21 and 22). The photograph shown in figure 21 is taken from Series X 8 B, slide 2, row 2, section 8. Here there is considerable abnormality in the ventral half of the labyrinth, and the labyrinth is correspondingly reduced in size. The anterior and posterior canals and the endolymphatic sac are fairly normal. In figure 21 can be seen the endolymphatic sac with its usual relation to the chorioidal membrane. The



section also passes through the anterior canal just oral to the crus commune and through the vestibular pouch. The latter is much reduced in size. It possesses a macula with a diminutive nerve and ganglion, but the lagena is absent. The lateral canal exists as a lateral pouch projecting from the vestibule (fig. 22). In spite of the abnormal shape due to the deficient vestibule the planes of the anterior and posterior canals intersect at the usual angle.

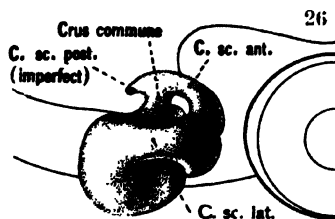
Labyrinth XII: (figures 23 and 24). The photograph shown in figure 23 is taken from Series X 7 C, slide 2, row 3, section 8. The section passes through the endolymphatic sac, the crus commune, the vestibule with the large pouch extending laterally from it and representing the lateral canal. The endolymphatic sac bears the usual relation to the chorioidal membrane. The anterior and posterior canals are fairly normal. The vestibule is enlarged and irregular, and illustrates the dropsical type. It possesses the usual macula with nerve and ganglion (fig. 23). The lagena is absent.

Labyrinth XIII: (figures 25 and 26). The photograph shown in figure 25 is taken from Series X 4 C, slide 1, row 3, section 5. It passes through the crus commune, the vestibule with its thickened floor, and through the lateral canal just being pinched off from the vestibule. Slightly oral to this the separation becomes complete. On examination of the reconstruction of this labyrinth (fig. 26) it can be seen that the chief injury involves the dorsal part of the labyrinth, and as a result the whole labyrinth is undersized. The greatest defect is in the posterior canal, which is represented by a bud extending caudally from the crus commune. A similar one is seen in figure 28 and the same kind of defect in an anterior canal has already been seen in figure 20. The crus commune and the anterior canal are small though complete. The involvement of the median surface is shown by the absence of the endolymphatic appendage and the lagena.

Labyrinth XIV: (figures 27 and 28). The photograph shown in figure 27 is taken from Series X 6, slide 1, row 4, section 2. This labyrinth is of the dropsical or vesicular type. It is possible to recognize its general position but aside from the fairly



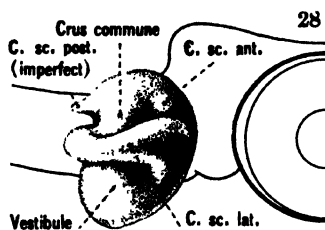
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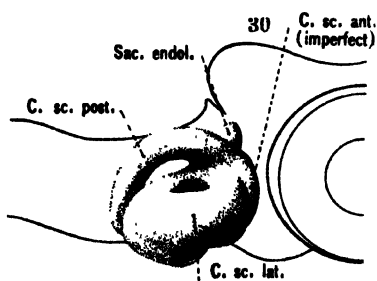
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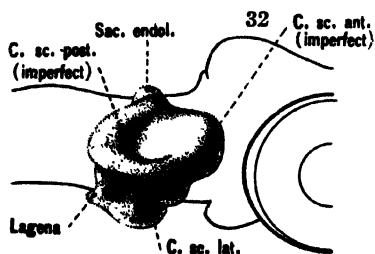
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normal lateral canal it is poorly differentiated (fig. 28). In this kind of a labyrinth after undergoing differentiation for a short time the process ceases. After that the only further sign of development is an increase in size, which thus produces the dropsical appearance, such as is seen in figure 27. The section is cut obliquely through the oral end of the lateral canal and the anterior canal. This section was chosen because it shows the macula and its nerve ganglion connection with the brain. The anterior canal, like the crus commune, is only slightly separated from the general vestibular cavity. The posterior canal is represented by a bud extending caudally from the region of the crus commune, such as we have already seen in figures 20 and 26. There is no definite sign of a lagena or endolymphatic appendage.

Labyrinth XV: (figures 29 and 30). The photograph shown in figure 29 is taken from Series X 1 A, slide 6, row 3, section 8. It passes through the oral end of the labyrinth, showing the endolymphatic sac, the small anterior canal, and the oral end of the lateral canal opening widely into the vestibule. As can be seen in figure 30, the caudal part of this labyrinth is fairly normal. The oral end is quite imperfect, the parts being **small** and not properly differentiated. The anterior canal is **only a pouch** not completely separated from the vestibule. The lateral canal has a deep impression on its dorsal surface marking the beginning of a separation. This labyrinth has not completely recovered its normal posture. It is displaced forwards and is tilted partially forward so that the endolymphatic sac, instead of bearing the usual relation to the chorioidal membrane, is opposite the mid-brain and lies embedded in the proötic ganglion. Though considering its abnormal shape it is not far from having the normal posture.

Labyrinth XVI: (figures 31 and 32). The photograph shown in figure 31 is taken from Series X 4 B, slide 2, row 2, section 4. The section passes through the endolymphatic sac, which in the sections a little more caudal touches the chorioidal membrane in the characteristic way. The section also passes through the crus commune and the vestibule with the lateral canal opening into it. The lateral canal is quite perfect, but the anterior and poste-

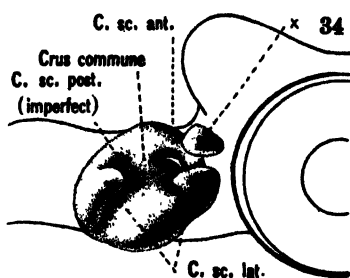
rior canals are only represented by pouches opening out of the vestibule in common with the *crus commune*. The shape of the pouches is relatively normal, and they are only deficient in not being pinched off from the vestibule. The lagena is quite normal. The acoustic nerve and ganglion are diminutive.

Labyrinth XVII: (figures 33 and 34). The photograph shown in figure 33 is taken from Series X 3 A, slide 2, row 3, section 13. Due to some mechanical or other injury the last three labyrinths in our series are quite imperfect, and from examination of the sections alone (figs. 33, 35 and 37) one would be quite unable to orient them. The reconstructions are of great assistance in this respect. The section taken from Labyrinth XVII was selected because it shows the 'labyrinth fragment' which evidently was a portion of the main vesicle that became detached and developed as a separate little sac (fig. 34, *x*). In figure 33 it can be seen outside the cartilaginous capsule, partially covered by a cartilaginous coat of its own. It is not connected either by nerve or duct with the main labyrinth. It is possible that in the course of the operation a fragment from the native vesicle was left in the operation-pocket, and eventually formed this structure. Examination of the reconstruction shows that there is an interruption in the lateral canal, its two ends being sealed off. One never finds an open end in these imperfect labyrinths, they are always sealed off. The anterior canal is small but well formed. The posterior canal with the *crus commune* from a common pouch communicating with the vestibule. The vestibule has a normal macula with diminutive nerve and ganglion. There is no lagena and no endolymphatic appendage.

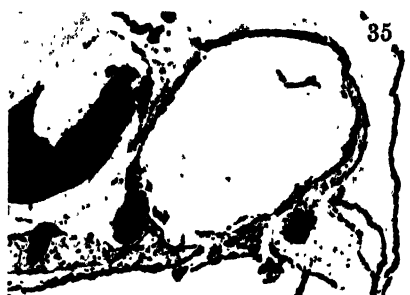
Labyrinth XVIII: (figures 35 and 36). The photograph shown in figure 35 was taken from Series X 3 B, slide 2, row 1, section 11. The whole oral region through which this section passes is of the dropsical type. The caudal region is not so pathological. The posterior canal, though incompletely separated from the vestibule and the *crus commune*, is otherwise well formed. The caudal half of the lateral canal is fairly normal, but the oral portion is distended like the rest of this portion of the labyrinth. Ventrally in one region this large labyrinth bulges through the parabasal



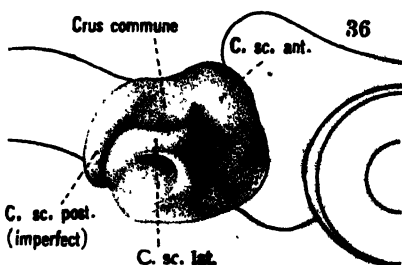
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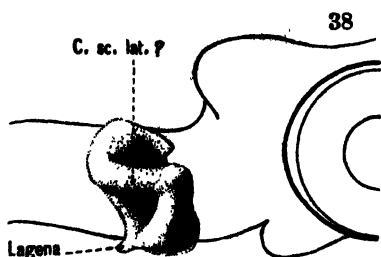
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plate and protrudes in the pharynx. On the lateral surface of the anterior canal there is a deep pit indicating the usual site at which it is pinched off from the vestibule and crus commune, otherwise the separation is lacking. The presence of a lagena is questionable, and the endolymphatic appendage is entirely lacking. This, like Labyrinth XVII, through quite abnormal, can be fairly definitely oriented.

Labyrinth XIX: (figures 37 and 38). The photograph shown in figure 37 was taken from Series X 11 A, slide 2, row 2, section 2.

This is the last and most imperfect labyrinth in our series. It must be regarded as only a fragment. Apparently there is a lagena with the characteristic finer structure, shape and position. There is also a canal that corresponds in form and position to the lateral canal. Otherwise the different parts cannot be very well differentiated. This labyrinth is included in spite of its fragmentary character because it shows that even such an imperfect labyrinth is apparently affected in its posture by the influences interacting between it and its environments.

In a summary of the first eighteen of these experiments the result that concerns us most is that in all instances where the transplanted labyrinth had developed with sufficient completeness for identification of its various parts it was found that it had almost perfectly regained its normal posture. It was this fact that formed the chief object of our investigation, but we may add the following concerning the resultant abnormalities produced by the manipulation of the vesicle at the time of the operation.

a. In 8 out of 18 experiments there developed a practically normal labyrinth;

b. Where a defect occurs following the operation it is usually localized in some particular region of the labyrinth; it may be confined to the anterior, posterior, or median portion, while the remainder of the labyrinth is quite perfect;

c. The three different canals are defective with about equal frequency (30 per cent), though in an individual labyrinth where the canals are involved the imperfection is not distributed among them equally; and the planes of the canals, whether they are defective or not, are usually normal;

d. Defects of the endolymphatic appendage occur with about the same frequency as those of the canals;

e. The lagena is imperfect more often than any other part (in 8 out of 18 cases);

f. The acoustic nerve and ganglion were always present, though in a few cases quite diminutive; the connection of the nerve with the brain wall can almost always be recognized.

DISCUSSION

The results that have just been recorded seem to show conclusively that there exists some influence between the transplanted ear vesicle and its environment that tends to control its posture, and that an inverted vesicle is thereby rotated back into the normal position. At the same time we must recognize the fact that Spemann ('10), from investigations of the same problem with experiments of much the same character, came to quite different conclusions. On examining and comparing our results, however, it will be seen that they are not necessarily contradictory, as thought by Spemann, but may perhaps be better described as differing in degree.

Including those in the present paper I have now reported 36 experiments in which the posture of the ear vesicle was especially studied. In 12 of these the ear vesicle had been rotated 180 degrees from its normal position; in 5 of them the ear vesicle had been transplanted without special placement; and in 19 of them the ear vesicle was transplanted and at the same time placed in a definite abnormal posture. In all of these 36 cases the labyrinth regained its normal posture. In Spemann's twelve reported cases in which there had been simple inversion of the ear vesicle, nine of the ear vesicles remained inverted, while two partially and one completely regained their normal posture. The latter Spemann regarded as an unsuccessful operation, surmising that it had slipped back into position directly after the operation. The fact of 'slipping' into the right position is the point of the whole matter. It is the remarkable fact that an ear vesicle, though rotated or turned or transplanted in an extremely abnormal position, nevertheless 'slips' into the correct position, that I am trying to establish in this paper.

It is of interest to examine Spemann's methods in search of some explanation for the difference in our results. For his experiments he used *Rana esculanta* larvae while I used *Rana pipiens*. It is not likely, however, that this would account for the difference in our results. His operations were performed at the time the ear vesicle is in the process of detaching itself from

the deeper layer of the epidermis. This is the same stage used by me. However, in the technique of the operation our methods are different. Spemann raises a relatively large quadrilateral flap of skin, thereby exposing the ear vesicle, which he loosens and inverts so that the anlage of the ductus endolymphaticus points downward. He then replaces the skin flap and secures it in position by a weight, consisting of a slightly curved strip of cover glass. There are two factors here that may account for the difference in our results. In the first place Spemann, by reflecting a large skin flap, exposes a larger area of the deeper structures and perhaps thereby injures the environment in a way that lessens the postural interaction between it and the ear vesicle. It will be remembered in my operations there was only a linear slit opened, which by the spreading of its edges was sufficient for the manipulation of the vesicle. In the second place the use of the weight, as is done by Spemann, to hold the skin flap in apposition may retard the movement of the vesicle and prevent its rotation. From the nature of my wound no weight was necessary.

In addition it may be mentioned that Spemann always placed his vesicles so that the median or convex side remained toward the brain, while in most of my experiments it was made to point lateralward toward the skin. In this respect the displacement of the ear vesicle in my cases was more extreme than in Spemann's, making it all the more difficult for my vesicles to obtain a normal posture. Furthermore, in Spemann's experiments the ear vesicles were replaced in their own original pocket, while in all of my last series they were transplanted to another specimen, here again adding to the difficulty of their postural adjustment. In all other respects there seems to be no essential difference in our methods.

Judging from my own experiments there certainly exists a decided tendency between the ear vesicle and its environment that serves to control the posture, though we know from Spemann's experience that under certain circumstances this tendency is interfered with and the necessary corrective rotation is not accomplished.

The development of a normally placed labyrinth from an inverted vesicle can only occur in one of two ways. Either the ear vesicle at the time of operation consists of indifferent cells which are capable of forming various parts of the labyrinth in accordance with how they chance to lie ('harmonisch-äquipotentiell System'); or the ear vesicle itself rotates as a whole after the operation, so that the cells originally intended for the different parts are brought to lie in their correct relation, where they continue in the fulfillment of their destined development. As was clearly argued by Spemann '10, it cannot be explained by the former. All our evidence points to a high degree of differentiation of the cells of the vesicle, and it is conspicuously proven by their possession of laterality, which has been described in the earlier part of this paper. We could not otherwise have a left-sided labyrinth on the right side of the head.

This leaves us with the alternative that the displaced ear vesicle does not stay in the position in which it is placed, but rotates into a normal posture. Regarding the nature of the force that produces the rotation there is yet little information. One must take into consideration at least three possibilities which either separately or in combination may explain its action. In the first place, it may be an active phenomenon on the part of the ear vesicle, that is, intrinsic motility of the vesicle itself; secondly, it may be based upon some attraction existing between some portion of the vesicle and the brain or other structure; and thirdly, there may be some purely mechanical basis for it.

In the first place, regarding the intrinsic motility of the ear vesicle itself, we are familiar with the flowing motion of protoplasm in the case of amoebal pseudopods, and Harrison ('10) has described the remarkable movements of the protoplasmic processes of nerve cells. In these instances there is a movement of one part of a single cell in relation to the rest of the cell. In the case of the ear vesicle, however, we should have to consider a mass-movement of a group of cells. Such movements have already been described for small masses of cells, such, for example, as the lateral line rudiment, which, as has been shown by Harrison ('03) migrates in the course of a few days, all the way from the head

region to the tip of the tail. Perhaps of a similar character is the shifting of groups of ganglion cells within the central nervous system as described by Kappers ('08) and the author (Streeter '08). These, however, are movements of small clusters of cells.

An example of intrinsic motility of a larger epithelial mass, which will be more analogous to the ear vesicle that we are dealing with, is afforded in the healing of skin wounds of young larvae. The epithelial coat, in such cases, spreads like an elastic sheet from the surrounding area over the wound. This mass-like movement of the epithelial coat is very characteristic. On watching the healing of a denuded area one can see the pigmented epithelial layer gradually spread in from all sides of the wound, covering in, as it does so, the exposed mesodermal elements, until eventually its edges tightly pucker together at the center. This is accomplished not by the formation of new epithelial cells but by the stretching out of the cells already there, as can be plainly seen by the alteration of the pigment pattern. The marked tension on the adjacent epithelium resulting from this movement in the region of the wound is strikingly shown by the way in which the pigment line existing along the dorsal crest of the larva becomes deviated toward the operated side. In such cases the epithelium belonging, we will say, to the left side of the body, is drawn well over to the right side, thus indirectly aiding in the covering of the seat of operation. For an excellent description of the behavior of the epidermis in the healing of wounds in larval *Necturus*, the reader is referred to the paper of Eycleshymer ('07). If the epithelial ear vesicle can move and adjust itself to varying conditions in the same way that the skin epithelium moves and adjusts itself we could then understand the rotation of the vesicles recorded in our experiments.

A second possibility is that the nerve and ganglion mass may serve to draw the vesicle into its proper position. At the time of the operation some of the ganglion element is usually transplanted with the vesicle, and as the latter develops we find the ganglion closely attached to the thickened part of its floor which is to form the macula. At the same time fibers have grown out from the central end of the ganglion to attach themselves to the

side of the brain wall. This tendency to an early nerve-ganglion connection between the ear vesicle and some portion of the brain wall is evident even when the vesicle is transplanted in a strange environment such as in front of the eye (Streeter '06). The nerve seeks the brain wall, and is it conceivable that after it is securely attached it would act as a check or guy rope on the vesicle. With the subsequent growth and the change in the relative positions of the different structures, the nerve attachment would pull on it and, depending on its previous position, would tend to rotate it one way or another. When the vesicle is in front of the eye there are so many abnormal factors present that one could **not** expect a successful correction of posture through this means. **But** in the auditory region where other things are favorable it is conceivable that the nerve attachment might at least help in the adjustment of the position of the vesicle. As a modification of this idea it is conceivable that there exists some mutual attraction between the macula, ganglion, and brain wall which tends to draw them together by some physico-chemical process. This would result in the same effect on the labyrinth as a whole as a tension of the acoustic nerve, and would serve in the same way as an assisting force in the adjustment of the position of the vesicle. In an analogous way it is possible that the endolymphatic appendage may be attracted to the side of the brain wall and thus tend to draw the vesicle into position. Wherever the endolymphatic duct and sac are well formed there exists a constant relation between them and the brain which I have never heretofore seen mentioned. The endolymphatic sac always lies closely applied to the membranous roof of the fourth ventricle near the rhombic lip. Some force may bring these two structures together, and thus we would have a secondary correcting influence on the posture of the whole vesicle. This, it must be admitted, cannot be the whole explanation, as we have vesicles with correct posture in cases where the endolymphatic sac is entirely absent.

As a third possibility there is to be considered, from a purely mechanical standpoint, the shape of the vesicle and the bed or pocket in which it fits. The auditory pocket is bounded on the median side by the relatively firm brain wall, ventrally by the

developing cartilaginous skeleton, laterally and dorsally by the non-resisting auditory capsule and skin. In front of the proötic ganglion mass and the optic vesicle, and caudally the vago-glossopharyngeal complex. These different structures present different degrees of resisting pressure, and thus from different directions there are these compression forces acting upon the ear vesicle, to which, when normally placed, it is properly adjusted by its shape and the firmness of its different parts. Normally there is an equilibrium between the compression forces of the environment and the resisting forces of the vesicle. When the vesicle is abnormally placed there is a disturbance in this equilibrium which continues until the vesicle regains its normal position. Thus we would have the mechanical tendency for the disturbed vesicle to fit itself into the right position. An objection to this explanation immediately suggests itself, and that is the fact that vesicles having an abnormal form, and that could not possibly fit well in the usual pocket, notwithstanding, right themselves almost as well as the normally shaped ones. As another mechanical factor one might think of gravity. It is well known that gravity constitutes a decisive factor in certain embryological processes. Hertwig ('99) and Wetzel ('04) experimentally produced deviation from the normal development of the egg by alterations in gravity through the use of a centrifugal machine. It is true that gravity controls the position within the egg membranes of the amphian larvae in the early stages. The ventral side, due to the yolk mass, is heavier and is always down. In the case of the ear vesicle the large macula in its floor is thicker and presumably heavier than the other portions of the vesicle wall, and we might assume this fact as the reason that we always find the macula towards the ventral side and thereby a factor in the posture of the vesicle as a whole. However, when the larvae are removed from the membranes, as is necessary for purposes of operation, the conditions are quite altered. Being no longer supported by a gelatinous sphere which is easily kept properly erect by gravity, the larvae fall to the bottom of the dish and rest on their side, and we immediately have an abnormal direction of gravity, which persists throughout the critical period in the development of the ear ves-

icle. If gravity were the controlling factor all of the ear vesicles in larvae removed from their membranes would be obliquely placed, whether operated upon or not. This we know does not occur, and therefore we may safely assume that gravity does not exercise any great influence on the posture of the ear vesicle.

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MULTIPLE FACTORS IN MENDELIAN INHERITANCE¹

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HISTORICAL

Since cases of simple Mendelian phenomena have been very frequently described, the interest of the student of genetics has shifted from the attempt to prove, or disprove, Mendelism towards the investigation of the extent of the application of Mendel's fundamental principles. Perhaps this is why the apparent exclusion of anything Mendelian from size inheritance by Castle's work on the ear length of rabbits, aroused so much interest. Castle ('09) found that the ear lengths of rabbit offspring were in general intermediate in relation to the parents. This seemed to indicate a simple blending inheritance in which the size-controlling elements from either parent were permanently joined in the offspring, never to segregate. Yet in the same rabbits Mendelian ratios were being given and segregation was taking place in the color of the hair. The appearance of a paper by H. Nilsson-Ehle ('09) brought a new possible interpretation of the ear length crosses. In this paper evidence was presented to show that there may be two or more Mendelian factors for the same character, which factors develop the character whether they appear alone or in any combination with the others.

Nilsson-Ehle found in certain crosses of oats and wheat in which the colors of the glumes and seeds were considered, that simple Mendelian ratios (3 : 1) were generally given, but in cer-

¹ This paper is based on an investigation carried on in the Laboratory of Genetics of the Bussey Institution with assistance from the Carnegie Institution of Washington. A full report of the investigation has been submitted to the Carnegie Institution for publication. This statement of some of its main features is published by permission. For the conclusions drawn from the facts recorded the author is alone responsible.

tain strains he obtained consistent ratios which indicated di- and tri-hybrid crosses ($15:1$, $63:1$); in other words, there were two or three factors involved, each of which alone or in combination with the others, produced the same color, and only in the absence of all such factors could the recessive color be seen. The application of this theory to the case of the rabbit ear lengths was immediately made, East ('10), Castle ('11), Lang ('11), by supposing that ear length depended upon several factors each of which behaved as a simple Mendelian unit, but lacked dominance. This would mean that we could see the difference between an ear whose length had been based upon four factors, and one whose length depended upon five or six factors. It would be supposed that the more simplex factors, or doses, there were present, the longer the ear. If the long-eared race bore four duplex factors (8 doses) for length, and the short-eared race bore two similar but not allelomorphic factors (4 doses), the hybrids obtained by crossing these two races would each have six factors in a simplex condition (6 doses). The relative sizes of the two parents and the hybrids would be expressed by the number of doses in each case. The second generation would give rabbits with various numbers of doses and their ears would range all the way from the length of the long-eared race to that of the short-eared race. Unless the two factors in the small race were allelomorphic to two of the four in the large race, there could be found rabbits with ears longer than the long-eared race, and others with ears shorter than the short-eared race, for in the second generation would be formed combinations involving as many as 12 doses and others with none. There would, however, be a much greater number of rabbits with intermediate than with extreme ear-lengths. Just as in a mono-hybrid cross without dominance there are two heterozygous individuals to one of each of the pure types, so in a multo-hybrid cross, where each factor, independent of the others, is giving a $1:2:1$ ratio, the individuals with all the factors in a simplex or heterozygous condition would be most numerous. The higher and lower grades would appear in decreasing frequency as the extremes were approached (Tammes '11). Now if only a few animals were raised in F,

it would be expected that they would have intermediate ear lengths. The numbers obtained by Castle were small enough not to show the new combinations expected on this theory.

On account of the many external conditions that may influence the development of the hereditary size factors, one could not expect to find the various grades in F_2 clearly enough separated to discover the number of factors that might be involved. However, evidence favoring a multiple factor interpretation of size would be found in the appearance in F_2 of extremes not found in F_1 or the increased variability of F_2 over F_1 , especially if extremes beyond the parental races appear.

In plants numerous experiments favoring a multiple factor hypothesis have been described. Since definite ratios may be obtained for qualitative differences, such characters afford the most convincing evidence. Supporting Nilsson-Ehle's results, East ('10 and '11), and East and Hayes ('11) in endosperm and pericarp colors of maize, found ratios which are apparently to be explained only by multiple factors. Tammes ('11) crossed two different sorts of blue Linum; an intermediate first generation was followed by a second generation with a wide range of variability, the intermediate shades having the greatest frequencies.

From quantitative differences in color one is led to quantitative differences in shapes and forms. The work of Kajanus ('11 and '12) on the roots of Brassica and Beta affords evidence to support the hypothesis that various form types of these roots are due to combinations of multiple factors for length, or length and roundness. Emerson ('10) reports increase in the variability of the shapes of the second generation from certain crosses with squashes and gourds. These cases of form differences do not show dominance. Shull ('11 b) described a case involving a double gene for the same character, that is dominant. He found that the flat and triangular seed capsule of *Bursa bursa-pastoris* is dominant to the oval and, in section, round seed capsule of its recent mutant *heegeri*. The ratios of the second and third generations strongly indicate that there are two genes involved, each of which can produce the *pastoris* type of capsule.

From quantitative differences in form we come to quantitative differences involving size alone. The possible application to size inheritance of this theory of multiple factors, so strongly indicated by color characters, was soon realized by Nilsson-Ehle ('07). He found that crosses between races of wheat or oats with long and short stalks gave intermediate hybrids whose offspring had stalks ranging all the way from the length of the long-stalked race to that of the short-stalked race. The length of the head of a certain wheat acted in crosses as though it were dependent upon a strong dominant shortening factor and two weaker factors for length, that lacked dominance. Spillman ('02) in crosses involving the length of wheat heads, and Emerson ('11), in certain crosses involving the height of corn plants, report intermediate first generations followed by second generations in which the ranges of variability included, or even exceeded, the extremes of the parental races. East and Brown ('11) present crosses of maize involving ear length, number of rows per ear, size of grain; Emerson ('10) sizes of beans; Tammes ('11) sizes of *Linum* seeds and petals; in all these cases the same characteristic results were found, namely, a constant and intermediate first generation and a second generation with wide variability. Philips ('12) gives a preliminary report on size crosses in ducks which indicates a like increase in the variability of the second generation birds.

Certain structural characters have been found that seem to depend upon two or more similar units of inheritance. In oats the presence and absence of ligulae, and the arrangement of the spikelets in the head—either on all sides of the rachis or only on one side—give strong evidence of multiple factors, definite ratio being obtained (Nilsson-Ehle '09).

Such physiological characters as winter hardiness, rust resistance and flowering times of cereals have been found by Nilsson-Ehle to show increased variability in crosses. Tammes ('11) found similar results in the opening or remaining closed of ripe *Linum* capsules. They partly open in the first generation; in the second generation some remain closed, others fully open, while most are half opened. Tschermak ('11) and Leake ('11) pre-

sented studies on the blossoming times of peas and cotton. In both plants crosses between late and early blooming varieties gave plants with intermediate blooming time. In the second generation some plants began to bloom as early as the early blooming variety and others as late as the late blooming variety.

Perhaps as strong evidence for the assumption of different factors influencing the same character as is afforded by ratios in the second and third generations, is found in crosses between apparently similar races which give marked variability in the second generation following a first generation that was like the two parents, and no more variable than the parental races. In such cases the characters in the two lines that have been crossed are supposed to be caused by different factors which produce the same effect. In the second generation, where recombinations first have a chance to appear, new grades are found. Cases have been mentioned that show extremes beyond the parental types. In these cases there must have been a different number of factors in each parental race, if we suppose for the moment that the factors are all of equal power. In the following cases each parental race may be supposed to have the same number of factors (although not identical ones) and so the races appear to be alike.

The occurrence of two white races of sweet peas which give colored offspring when crossed is undoubted evidence that two different factors are involved in producing color. In the following cases the factors are supposed to be similar to each other so that their presence cannot be determined until the second generation. Nilsson-Ehle ('11) found two strains of red wheat, which, when crossed together gave whites in the second generation. Whites also appeared when certain lines of black oats were crossed, in the ratio of fifteen blacks to one white. To lines of oats (Nilsson-Ehle '09) whose spikelets were arranged on all sides of the rachis gave, in the second generation, some plants with spikelets on one side of the rachis. Kajanus ('11) reported three crosses between apparently similar races of long beets which gave the same form in the first generation, while in the second generation some very long individuals were obtained. In crossing dif-

ferent strains of cotton of equal height Balls ('07) found a great variability in the heights of the second generation. The first generation was uniformly close to 100 cm., the second generation ranged from 30 cm. to 100 cm. Hayes ('12) has crossed varieties of tobacco with similar numbers of leaves which produced plants with the same number of leaves in the first generation. The leaf number for the plants of the second generation was very variable. Keeble and Pellew ('10) have worked out a very clear explanation of an increase in variability found in a second generation following the crossing of two semi-dwarf races of peas. The parental races were very constantly between 3 and 4 feet tall. The first generation showed increased vigor, but a constant height of 7 to 8 feet. The second generation ranged from $1\frac{1}{2}$ to 8 feet. This case differs from those preceding in that the factors, to whose recombination the new grades in the second generation were due, did not produce the same effects. One factor increased the distance between the nodes, the other increased the thickness of the stem, and this thickening enabled the internode to attain greater length. One parental race had the factors for thick stems and short internodes, the other had the factors for thin stems and long internodes. The recombinations in the second generation produced plants with long internodes and thick stems (8 feet as well as plants with short internodes and thin stems ($1\frac{1}{2}$ feet). The combinations found in the parents also occurred. Moreover, the ratios in the four classes closely approximated expectation. In this case height is dependent upon two Mendelizing factors.

In other cases size may depend upon a single factor. Some of these cases are mentioned: tall and dwarf peas, Mendel; tall unbranched habit vs. dwarf branched sweet peas, Bateson and Punnett ('08); axial vs. terminal position of bean pods, Emerson ('04); tall vs. dwarf *Antirrhinum*, Baur ('11); dwarf vs. normal tomatoes, Drinkard ('08); long vs. short styles of *Oenothera*, de Vries ('01, p. 435); long vs. short wings of *Drosophila*, Morgan ('11); long vs. short hair in various mammals, Castle ('05); brachydactylous digits in man, Farabee ('05) and Drinkwater ('08);

short stocky vs. long slender legs of Dexter-Kerry cattle, J. Wilson ('09).

The evidence presented by plant breeders seems to carry conviction. It has shown that the assumption of multiple factors is the most simple theory to explain certain ratios in crosses involving color and its absence. Similar ratios are also found in crosses involving certain structural characters (ligulae and the arrangement of spikelets in wheat). Now if qualitative characters may depend upon multiple factors in certain cases, there is just as good evidence for saying that certain morphological characters depend upon multiple factors. Size crosses differ from crosses involving multiple factors for color and structural characters in that in the latter cases the color or the structure may be absent, and, so definite ratios can be found; whereas in the former size as such cannot be absent, so in most cases no definite ratios can be found. Otherwise, these two types of characters are strikingly similar. Both show wide variations in the second generation not found in the parental or first generations. Both types show similar wide variations in the second generation in crosses between certain strains that appear to be alike and in these cases the first generation is like the parents. Both types may show mono-hybrid ratios in the same sorts of characteristics. Any theory to account for the wide variations in the second generations of crosses involving characters which cannot afford definite ratios must also account for this similarity with cases where definite ratios may be found, and again, with cases where simple Mendelian ratios and complex ones are found for the same character differences. Size and Mendelian inheritance are not incompatible, as is shown by many mono-hybrid ratios from crosses involving size. In one case (semi-dwarf peas) it has been shown even by ratios that size may depend upon two distinct Mendelian factors. Surely the most simple theory that can be given to explain this phenomenon of increased variability, which in itself cannot be doubted, being reported by many investigators for so many characters, is that there exist units of inheritance, introduced by the parents, to the segregation of which units in the germ cells of the hybrids, the new combinations are due.

EXPERIMENTAL

In 1908 Castle started a second experiment that should test this hypothesis by obtaining larger numbers of animals so that the variabilities of the first and second generation could be compared. As the size of the whole animal was to be considered, crosses were made between a small male rabbit from a small race, the Himalayan, and a series of large females that had been used in the ear length crosses. The pedigrees of the females are known and for several generations there were only slight differences in the weights of the pairs of ancestors. In 1910 the experiment was put into the hands of the writer. By that time the original crosses had been made and weights for growth curves had been recorded for the animals raised up to that time. I wish to acknowledge here a keen appreciation of my indebtedness to Professor Castle for the privilege of completing this work which he had planned and already started, and to express gratitude for the assistance and advice that he has contributed towards the completion of this work.

In most cases the second generation consisted of a back cross of the first generation females to their male parent. A few back crosses of first generation males to their female parent were made and a few crosses between first generation males and females. Measurements were made of the skull and long bones of the rabbits after the bones had attained full size. Fifteen months was set as the age for killing a rabbit, as it was found that the bones were fully grown at that age. Sixteen measurements for each set of bones were recorded.

a. Coefficients of size

It was found that there was enough lack of correlation between the various measurements to give different results when different characters were considered, so it became needful to obtain a number for each animal in which the various measurements would be equally represented to express the size of each animal. As the measurements varied from 2 to 10 cm. no absolute average could be used; for a small deviation in a short measurement

would have far greater significance than an equal deviation in a long measurement, and so in any average the large deviations in the long measurements would entirely overbalance the equally important small deviations in the short measurements. The following method was used to obtain a number for each animal, which may be called the coefficient of size (C. S.), a number based on relative deviations. The average of a character for one fraternity was used as a dividend into which were divided the individual measurements of the animals in the same fraternity. The quotients so formed gave a series of ratios expressing the relative sizes of the various sibs above or below their mean. By this method the ratios of the other characters of one animal to the corresponding fraternal means were obtained. The average of these ratios gave the coefficient of size (C. S.) for that animal. These coefficients range from 0.930 for the largest animals to 1.070 for the smallest ones. In classifying them, classes 0.005 in extent were used. From these distributions, standard deviations were calculated. Since the means of all fraternities lie always in the column whose value is 1, it will be realized that the sums of all the individuals in each column will give a correct description of the variability of one generation of a whole family; in other words, different fraternities from the same family may be classified together. The standard deviations of these family distributions will be based on deviations from the various fraternal means. This will give a more accurate result than if the actual deviations had been calculated from the means of all the individuals averaged together, as would be done in applying the formula for standard deviation to the values themselves. It must be supposed that the means of all the fraternities do not coincide. The mean of all the values would give the extreme variates even greater deviations and the standard deviations would be higher than those obtained by this method. It will be seen now that the coefficients of size of all the animals in one generation may also be classified together.

In the tabulation of these coefficients of size it is at once observed that all the most extreme coefficients belong to individuals

from the back cross. Naturally both the F_1 and the back cross offspring occur in greatest numbers about the middle class and decrease in either direction away from this class. But the back cross extends much further than the first generation. It is unfortunate that the numbers of the F_1 are very much smaller than those of the back cross. It may be claimed that with larger numbers more extremes would have been found in the F_1 . This is undoubtedly true, but it is highly improbable that any individuals that approached the back cross extremes would have

TABLE 1

Standard deviations of coefficient of size of the first (F_1) and back cross (B.C.) generations in the various families. The numbers of individuals are given for each fraternity (No.).

FAMILY	F_1	NO.	B.C.	NO.
647	0.0145	4	0.028 ± 0.002	25
1443			0.026 ± 0.003	17
1471	0.015	7		
1491	0.015	4	0.019 ± 0.001	24
1493	0.009	13	0.021 ± 0.001	60
1531			0.027 ± 0.001	45
1532			0.027 ± 0.003	15
1537			0.020 ± 0.001	46
2011	0.019	5	0.025 ± 0.003	11
1493 ¹ \times 2379	0.019	14	0.017 ± 0.001	31
Total.....	0.014 ± 0.001	33	0.023 ± 0.0007	243

¹ This family is not from a size cross. Is is used as a check, so is not included in the totals.

been found. The standard deviations show that there is a real difference in the variability of the two generations. In table 1 the standard deviations in terms of the coefficients of size are given for the two generations, one family at a time as well as for the totals of each generation. In every family resulting from a size cross the standard deviation of the back cross exceeds that of the first generation. Family 1493 \times 2379 was carried along as a control as the parents were nearly the same size. It will be noted that the standard deviation of the back cross, in this case,

is slightly smaller than that of the first generation. In families 1443, 1531, 1532 and 1537 there were not enough F_1 individuals to use in determining coefficients of size. The back crosses of these families are given for comparison with the first generations and back crosses of other families. In family 1491 the standard deviation of the back cross is the same as the highest first generation standard deviation, namely, that in family 2011. In 1493 and 1537 the back cross standard deviations are very nearly as low as the highest first generation standard deviations, but the standard deviation of the first generation of family 1493 is much lower than that of the back cross of the same family. This is the largest family obtained.

b. Classification in relation to the parents

The data were next treated in a way to show graphically the size relations between the parents, the first, and back cross generations, considering one character at a time. This method of treatment also shows the relative variability of the first and back cross generations and offers another method for combining different families. This method may be called classification in relation to the parents. For each family 15 classes were formed between the parental classes. Each parental measurement is taken as the middle of a parental class; the extent, or range of each class equals one-sixteenth the difference between the parental measurements. Into classes so formed the first and back cross generations were separated. This was done for all characters in all families. From these classifications the number of animals of all families that fall half way between their parents in regard to any measurement is readily found by adding the frequencies in the middle column; likewise the sums in other columns show the distribution of animals in other relations to their parents. The male parent in all families was the same animal. The female parents differed in size to some extent. This means that in the various families and in the totals, individuals are put together that are not exactly similar as to size. They are, however, similar as to their relative positions between their respective parents.

In general the size of all the measurements of the first generation is plainly intermediate, although the modes and means of the various frequencies are markedly above the midparental. This high distribution may readily be interpreted as the effect of increased vigor from the cross. Similar plant crosses show a like increase in size in the first generation (see East '09, Shull '09, Darwin '76). A marked difference in variability is seen in these first generation frequencies when different characters are compared. When the range is wide it is as much extended in one direction as the other, showing that the extreme lows are not to be accounted for by underdevelopment, as an asymmetrical variation might suggest. Such a difference is found when the distribution for the ulna, which has a wide range, is compared with the distribution of the skull length, which has a very limited range (table 2). Four of the remaining measurements are similar to the skull length; two are intermediate; six are wide, slightly less than the ulna. Yet these frequencies include the same individuals. Pearson ('02) found similar inequalities in different parts of the human body. Hatai ('07) found greater variability in the length of the nasal bone and in the zygomatic width in skulls of albino rats than in any other skull characters.

The variations in the ranges of distributions of the different characters, described for the first generation, are also found in the back cross. The means of the back cross fall near the class half way between the midparental (middle class) and the small parent to which the first generation females were back crossed. A very marked feature of these back cross frequencies is the number of measurements that are as low and lower than the small parent, and as high as, and higher than the mode of the first generation frequencies in which their mothers are included. Since the father and grandfather in all the families was the same rabbit, the actual values of the male (small) parental class in all families is the same, and the values of the adjacent classes differ very slightly in the different families. This means that all animals falling in classes near his class have very nearly the same sizes in the different families. This makes the occurrence of these short measurements the more convincing evidence of segregation.

TABLE 2
Classification in relation to parents based on skull lengths and ulna lengths, to show the relative variability of the two measurements and of the first generation (F₁) and the back cross (B. C.)

[illegible]

With assistance from Mr. S. Wright standard deviations were calculated for the total first generation and back cross frequencies, as classified in relation to the parents, for each measurement (table 3). These constants do not give actual deviations because the totals are classified in a purely relative manner. However for purposes of comparing the two generations these standard deviations are permissible because the frequencies in both generations are classified in relation to the same parental classes.

TABLE 3

Means and standard deviations of the first generation (F_1) and back cross (B.C.) based on classifications in relation to the parents, similar to those given in table 2 and expressed in terms of those classes.

MEASUREMENT	MEAN F_1	MEAN B.C. σ	σF_1	σ B.C. σ
skull length, total...	10.80	5.55	1.94 ± 0.15	2.60 ± 0.08
skull length, partial, 1.	10.56	5.24	2.76 ± 0.21	2.69 ± 0.08
skull length, partial, 2.	10.02	5.28	2.50 ± 0.19	2.80 ± 0.09
skull width, anterior...	8.39	2.01	3.17 ± 0.24	3.52 ± 0.11
skull width, posterior...	11.00	2.17	3.63 ± 0.27	4.58 ± 0.14
teeth.....	10.12	5.69	2.56 ± 0.19	2.88 ± 0.09
nasal.....	9.20	4.35	3.47 ± 0.26	2.87 ± 0.09
mandible 1.....	10.27	4.75	2.04 ± 0.15	2.99 ± 0.09
mandible 2.....	8.95	3.16	2.90 ± 0.22	3.29 ± 0.10
humerus.....	12.20	5.86	3.65 ± 0.27	3.55 ± 0.11
ulna.....	12.24	5.27	5.43 ± 0.42	4.61 ± 0.15
femur.....	11.83	5.54	3.85 ± 0.30	3.38 ± 0.10
tibia.....	11.90	6.16	4.07 ± 0.31	3.98 ± 0.12

Six measurements show significant increase in the back cross over the first generation, three show decrease in the back cross, and four lie within the probable error, meaning practically no change. This is a mathematical demonstration that there is enough lack of correlation between the bone measurements for different characters to give different results. Since twice as many characters show increase as decrease, it can be concluded that there is greater variability in the back cross offspring than in the offspring of the first generation.

c. Body weights

When the work was taken over by the writer it was believed that body weight could be taken as a measure of size. Accordingly weights of the rabbits were recorded weekly. From these records growth curves were plotted by which the adult weight for each animal was to be determined. That this could not be done with any nicety, was clearly demonstrated by the study of some 300 curves. Rabbits' weights are very sensitive to changes in conditions; and to obtain curves that would be smooth enough to determine adult weights with accuracy would require more perfect experimental conditions than it has been possible to obtain in raising large numbers of animals. In many cases fat is deposited in such a way that there is no flattening of the curves at about 150 days, as in most cases; instead it may continue to rise for a year without flattening. Pregnancies and nursing disturb the curves of the F_1 females.

The most important information the growth curves afford is their vouching for the recovery of animals that have been sick. In spite of fluctuations, one can see in nearly every one the trace of a regular curve. Through a recognition of the normal type of curve it soon became possible to determine whether at a certain point in a curve an animal was above or below its normal. Based on this element of regularity, which, when shown by a part of a litter, gives the type of curve the other would be expected to have followed, adult weights were estimated. Approximations were made within 100 grams; in a few cases within 50 grams. Coefficients of variability were calculated for the F_1 and back cross fraternities (table 4). Since the means were absolute, standard deviations could not be used in making comparisons. In ten fraternities the C. V. of the back cross animals are higher than any of those of the first generation fraternities. In five fraternities the coefficients of variability are lower than the highest coefficients of the first generation. The weights show, then, that greater variability is found in many of the back cross fraternities than in any first generation fraternity. Whereas by themselves

TABLE 4

Coefficients of variability of weights, arranged by generations. Each fraternity is considered separately in the back cross (B.C.)

FAMILY	C. V. of F ₁	no.	C. V. of B. C.	no.
647	5.16	4	10.52	5
			5.58	6
			10.27	7
1471	4.09	7		
1491	6.22	4	10.46	10
			6.32	7
1493	8.75	15	13.56	8
			8.30	8
			9.29	12
			9.94	6
1531			13.46	16
			10.45	14
1532			10.27	10
1537			7.35	10
			8.23	13
2037			10.83	23

these weights might bear little or no conviction, on account of the roughness of their estimation, as cumulative evidence in connection with the results based on bone measurements, they certainly may be considered.

CONCLUSION

The conclusion to be drawn from these observations seems clear. The back cross is more variable than the first filial generation. This appears in the relative distributions of the coefficients of size of the two generations, whether compared by observation or by standard deviations: it is found when the two generations are classified one character at a time, in relation to the original parents, whether single families or whole generations are considered: and finally the coefficients of variability of the estimated body weights support the same conclusion. There occur characters among the back cross offspring that are smaller than the corresponding characters in the small parent and others that are larger than the modes of the first generation large parents.

This conclusion is very similar to many of the cases cited above. All of these deal with heritable characters quantitatively different. They are subject, to a greater or less degree, to fluctuations that, not being heritable, may be roughly assigned to environment. Offspring from crosses between extremes are generally of an intermediate nature. In the following generation new forms appear that are similar to the original parents or even more extreme. The greater number of individuals are intermediate. In certain cases crosses between similar lines, after a first generation like the parents, give a second generation in which a wide range of grades appear. These are the facts that can be definitely ascertained from the work that has been done. On the probability that these same phenomena will always be found, a law may be stated: the second generation of a size cross will show greater diversity than does the first generation or the parental lines. All practical application will come from this.

The interpretation of multiple factors can be applied to all the facts. It goes hand in hand with the mutation, and pure line doctrines of de Vries and Johannsen, and in its breadth of application, and its comprehensive simplicity, this theory, based on the assumption of the segregation of distinct units, is very attractive; by its use as a working hypothesis important facts have been discovered; its acceptance and further development will help to establish a broad and unified system of heredity.

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THE REACTIONS OF NORMAL AND EYELESS AMPHIBIAN LARVAE TO LIGHT

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TWO FIGURES

A great deal has been written about the photic reactions of amphibians, but this has been concerned chiefly with the adults, the larval forms having received but scant notice. In the spring of 1913 a series of experiments were carried out upon the larvae of *Rana pipiens*, *R. sylvatica*, and *Amblystoma punctatum* to determine whether they were sensitive to light, and if so, whether they were sensitive to light received through the skin as well as to light received through the eyes.

I take this opportunity to express to Dr. R. G. Harrison my thanks for suggesting this piece of work to me, as well as for his continued interest and criticism.

Banta and McAtee ('06, p. 71) have noted that the larvae of the cave salamander are much more responsive to light than are the adults, and that the younger larvae are more responsive than the older. Both larvae and adults are negatively phototactic. Eycleshymer ('08) found that *Necturus* larvae were negatively phototactic, both in their natural environment and in the aquarium; furthermore, that they orient in a definite way, such that the light falls with equal intensity upon the two sides of the body.

The only other mention made of the reactions to light of amphibian larvae is that by Franz ('10 and '13). Franz describes tadpoles as being indifferent to light—non-phototactic—except when they are crowded into a small space, under which conditions they will all orient themselves to the rays of light, so that their heads are directed toward the source of light. Franz cites this case as one of the numerous examples of so-called phototaxis,

which he has called a 'Fluchtbewegung,' caused by the abnormal conditions under which the animals find themselves.

My experiments were carried on in a basement dark-room, the temperature of which varied between 15° and 19°C. A 60-watt Mazda lamp on a 110-volt circuit was used as the source of light. This was placed in a wooden box, in one end of which there was an opening 8 cm. square, through which the light was projected. The larvae whose reactions were to be tested were placed singly in a flat cylindrical glass dish, of 22 cm. diameter, and 8 cm. deep, holding 2500 cc. of water. The sides and bottom of the dish were covered with black paper, except on the side toward the light where a small window, 3 cm. wide, and extending from near the top to the bottom of the dish was cut. The dish was placed so that its middle point was at a distance of 50 cm. from the lamp, at which point the light had an intensity of about 192 candle-meters. Between the lamp and the dish, and 15 cm. from the former, a screen with an opening 3 cm. high, and 1.5 cm. wide, through which the light passed, was set up.

The larvae used varied somewhat in size, but at the beginning of the experiments the tadpoles were about 12 mm., the *Amblystoma* larvae about 18 mm. long, none of them being shorter than 15 mm. During the course of the experiments, which were begun early in April, and continued through May and part of June, the larvae grew in size, until the tadpoles were about 20 mm., the *Amblystoma* 30 mm. long.

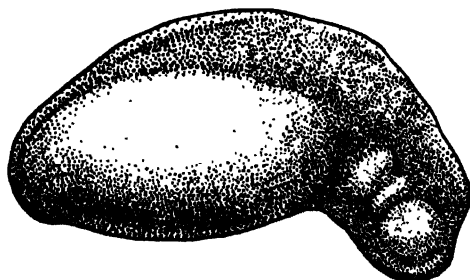
In order to find out whether the larvae were sensitive to light received through the skin, it was necessary to remove their eyes. The method used for removing the optic vesicles was that described by Lewis ('04, '05 and '07) and by LeCron ('07). The instruments used were a pair of very finely ground Noyes iridectomy scissors, and a fine pointed pair of forceps for holding the embryos. The stage at which the tadpoles were operated is that figured by Harrison ('04, p. 201) when the tail bud is just beginning to be perceptible (fig. 1). The *Amblystoma* embryos were operated at the corresponding stage (fig. 2). All the embryos were operated with a binocular microscope. They were placed in watch glasses in a 0.2 per cent normal salt solution, in

which they remained until the wounds had healed, after which tap-water was gradually added. When the larvæ had attained a certain size they were transferred to battery jars, each larva being placed in a single jar, which was numbered.

All the larvae tested were kept separate in small battery jars about half full of water and in which a few water plants of various kinds were placed. The frog tadpoles will feed upon the



1



2

Fig. 1. Embryo of *R. sylvatica*, to show the stage of development used in the beginning of the experiments (after Harrison). $\times 9\frac{1}{2}$.

Fig. 2 Embryo of *Amblystoma punctatum*, to show the stage of development used in the beginning of the experiments. $\times 9\frac{1}{2}$.

leaves of these plants but the *Amblystoma* larvae will not. The *Amblystoma* larvae were fed regularly during the course of the experiments on small crustaceans which they devoured eagerly, the blinded larvae seeming to have very little difficulty in seizing their quickly-moving prey. The jars were numbered, so that the reactions of the individual larvae could be followed. It was found necessary to isolate the *Amblystoma* larvae early for

the reason that when several are kept together in a single dish they will nip each other's gills, legs and tails, rendering themselves unfit for experimentation.

The larvae were tested in groups of ten, and each individual in a set of tests was given in order a single trial, until each one of the ten had been tested once. This was then repeated until each had had ten trials. A single set of tests, therefore, consisted of 100 trials. The larvae were always dark-adapted, being placed in the dark-room during the afternoon of the day before the experiments were made, and therefore subjected to a condition of darkness for at least fifteen hours before being tested.

Some little difficulty was experienced at first in placing and orienting the larvae in the dish in which they were tested. But after a little practice, it became comparatively easy by means of a large pipette to place a larva in the middle of the dish and at right angles to the impinging rays of light. During the ten reactions that each individual was given in a set of tests, care was taken that both sides of the larvae should be exposed to the light, but no attempt was made to expose the opposite sides in consecutive trials. A larva after having been oriented properly was allowed five minutes in which to react. If at the end of that time it showed no response, it was replaced by another.

It was soon found that the frog tadpoles, both normal and blinded, do not respond to stimulation by light. They were tested both in daylight and in artificial light, and in neither do they show any reaction. As Franz holds ('10 and '13) frog tadpoles are non-phototactic. Franz's experiments of placing a few tadpoles in a small dish and floating this on the water in a larger dish containing tadpoles which show no orientation to light was repeated. But in no case did these tadpoles show any orientation to the light, but continued actively swimming about in all directions, colliding with the sides of the dish and with one another. A single individual placed in the small dish behaved in the same way.

The *Amblystoma* larvae behave very differently. They are sensitive to light and respond to its stimulus by very definite movements. As has been mentioned, the larvae were given five

minutes in which to react, but the reactions were usually quick and definite, the reaction time varying between ten and one hundred and twenty seconds, the average being about fifty seconds. Sometimes a response did not take place for a longer time, but in the majority of such cases it was difficult to say whether the movement was made with reference to the light. Such reactions were therefore called indifferent, that is locomotion straight ahead, or nearly straight ahead, without apparent reference to the light. The typical positive or negative reaction consists, in the first place, of a turning of the anterior end of the body toward, or away from, the light. The turning is usually assisted by the fore-legs, though sometimes they are relaxed and are dragged passively, in which case the turning must be brought about solely through the contraction of the body muscles. This turning of the anterior end of the body is usually followed by a swimming, or creeping on all-fours, in the given direction. Sometimes, however, a larva remains bent in a curve for several seconds, with its anterior end in the given direction and with its posterior end still at right angles to the rays of light. In a few cases this constituted the entire response, the larva remaining in this position for five minutes. In a few other cases, always positive, the whole response is carried out so rapidly that no distinction between turning and swimming toward, or away from, the light could be made. There were, in addition, cases where a larva after having been oriented remained absolutely still for five minutes. In this event it was replaced by the next in order.

In the reactions of the same and different individuals there were the usual inconstancies which one observes in the study of the light reactions of almost any animal, and which have been referred to, for want of a definite explanation, as being due to the changing physiological conditions of the organism. In some cases an individual in a set of ten, on one day would be persistently negative, indifferent, or show no reaction, while two or three days later it would be as persistently positive. Again an individual might show no reaction within five minutes for one trial, and thirty minutes later it might be decidedly positive.

Although the results of the reactions of the individual larvae were kept separate, they were throughout so similar that there is no need to give them separately and they are given combined, in table 1. Five groups of ten larvae each were tested for the reactions of normal individuals, and four groups of ten each for those of the eyeless. It is seen, by referring to the table that, in the case of the normal larvae, out of a thousand reactions, 786 were positive, and 127 negative, while 54 were indifferent, and 33 gave no reactions within five minutes. In percentages these results give 79 per cent positive, 13 per cent negative, and only 5 per cent indifferent, and 3 per cent no reactions within five minutes.

TABLE I
Photic reactions of normal and eyeless Amblystoma larvae

REACTIONS	CONDITION OF LARVAE							
	Normal				Eyeless			
Directions of.....	+	-	±	0	+	-	±	0
Number of responses.....	786	127	54	33	706	150	79	65
Per cent of responses.....	79	13	5	3	71	15	8	6

The eyeless individuals are almost as decidedly positively phototactic as the normal are. Here there are 71 per cent positive and 15 per cent negative, a decrease of 8 per cent, compared with the normal larvae, in positive responses and an increase of 2 per cent in the negative. There is, however, a larger percentage of indifferent reactions and more of them do not react in five minutes, than in the case of the normal larvae.

These results show that the larvae of *Amblystoma punctatum* are positively phototactic in response to stimulation received through the skin as well as to stimulation received through the eyes and the skin. These results are in accord with what has been found by many investigators of the light reactions of the amphibians, that is, that the eyes are not essential for the light reactions, for the latter may be brought about by stimulation through the skin. Parker ('03), Pearse ('10), and Laurens ('11) have reviewed the literature of this subject, and there is

no need to do so again here. It has also been shown that the phototactic reactions of amphibia when the skin alone is illuminated are apparently not due to the direct stimulation of the central nervous system by light. Parker ('03) has shown this in the case of the frog, while Dubois ('90) on *Proteus*, and Reese ('06) on *Cryptobranchus* and *Necturus* have done the same for these animals. *Amblystoma* larvae are, of course, much smaller and more transparent than the adult forms which these investigators used, and the chances that the light, penetrating the muscles, might directly stimulate the central nervous system are much greater. But further series of experiments bring additional evidence that here again the reactions are not due to the direct stimulation of the central nervous system. In these experiments the testing dish was placed at a distance of 25 cm. from the light,

TABLE 2

Reactions of normal Amblystoma larvae to local skin illumination

REACTIONS	REGIONS ILLUMINATED											
	Head				Mid-body				Tail			
Directions of.....	+	-	±	0	+	-	±	0	+	-	±	0
Number of responses.....	123	31	22	24	107	26	36	31	110	32	31	27
Per cent of responses.....	62	15	11	12	54	13	18	15	55	16	16	13

where its intensity was about 768 candle-meters. The beam of light was made to pass through a small opening in a screen placed close to the glass dish. In this way a small area of a larva could be illuminated. A series of tests were made on ten normal and on ten blinded larvae, in which different small areas were illuminated. Three regions were selected which may roughly be described as the head region, the ventro-lateral mid-body region, and the tail region. Fifteen minutes was the time allowed for the responses. The results of these tests are given in tables 2 and 3. As may be seen, positive responses were obtained when each of the three regions in both normal and eyeless larvae were exposed to the light. A simple turning of the anterior end of the body toward or away from the light was taken as a positive or negative response. The beam of light was, of course, so small,

TABLE 3

Reactions of eyeless Amblystoma to local skin illumination

REACTIONS	REGIONS ILLUMINATED											
	Head				Mid-body				Tail			
Directions of.....	+	-	±	0	+	-	±	0	+	-	±	0
Number of responses.....	112	35	27	26	106	30	35	29	108	38	25	29
Per cent of responses.....	56	18	13	13	53	15	18	14	54	19	12	15

that a movement in its path toward or away from its source was impossible. The percentages of indifferent reactions and of no reactions within fifteen minutes were high as compared with the results obtained when the entire eyeless animal was illuminated. The head region in the normal larvae proved to be more sensitive than the others, due to the fact that the retina was also illuminated. In the blinded larvae there was no evidence that one region was more sensitive than the others, which is not in agreement with the results of other investigators on other forms. Dubois ('90) found the tail of *Proteus* to be the most sensitive region of the skin, and both Reese ('03) and Pearse ('10) found the same for *Cryptobranchus*. Reese also noted that the head of *Necturus* was more sensitive than the tail, due probably to stimulation received through the eyes, and Pearse showed that when the eyes of *Necturus* were removed the tail was the most sensitive region. Eycleshymer ('08) also found that in both normal and decapitated *Necturus* larvae the tail is the most sensitive region of the skin.

As has been seen the larvae of *Amblystoma punctatum* are positively phototactic, both in the normal and eyeless condition. The adults, on the other hand, are negative, both when normal and when blinded (Pearse '10, p. 173). We have here then an example of an animal changing the sense of its reaction to light from positive to negative with its change from the larval stage to the adult.

The skin chromatophores of amphibian larvae have been shown to present different conditions of expansion and contraction of their pigment under different conditions of light and darkness.

Babak ('10 and '13) has recently pointed out that normal and eyeless *Amblystoma* larvae show differences in pigmentation according to the illumination and background. The dark condition of the larvae, in which the pigment cells are expanded, can be produced in three ways, namely, (1) by placing normal larvae in diffuse light on a black background, (2) by placing normal larvae in darkness, and (3) by placing blinded larvae in diffuse light. The light, or pale condition of the larvae in which the pigments cells are contracted, can be produced (1) by placing normal larvae in diffuse light, on a white background, (2) by placing blinded larvae in darkness. It occurred to me that perhaps the condition of the pigment might affect the sensitiveness of the larvae to light, in that in an individual in which the pigment was contracted, the nerve terminations in the skin might be more easily stimulated than in one in which the pigment was expanded. A series of comparative tests were, therefore, carried out with the object of finding out whether there was any difference in the sensitiveness of dark and pale animals, and also whether adapting the larvae to darkness or to light affected their photic reactions. Two groups of normal and two groups of blinded larvae consisting of ten each were selected, and were exposed in turn to the several conditions of illumination and background mentioned above. Battery jars entirely covered with white paper were used to obtain a white background. Babak's results were verified. Flemming ('97) obtained somewhat similar results with normal salamander larvae. He found that if they are placed in darkness they become dark, and that if they are placed in bright light in white porcelain dishes, they become pale and transparent. Fischel ('96) obtained exactly opposite results with the larvae of *Salamandra maculata*, which he found to become pale when kept in darkness, and dark when kept in the light. Fischel's results agree with those of Hermann ('86) on frog larvae, a condition exactly opposite to what is found to take place in the adults.

In my experiments the dark-adapted larvae were kept in the dark five to seven days before they were tested. The larvae which were light-adapted were allowed to remain three to five days in bright diffuse daylight, and were tested only in the after-

noon in order that the light might have had as strong an effect immediately before the experiments as possible. The jars in which the larvae were kept, were placed on a broad window-sill of a room into which the dark-room opened, and a larva could be placed and oriented in the testing dish within twenty seconds of the time that it was first picked up.

The results of this series of tests are shown in tables 4 and 5. It was soon found that the condition of the pigment in the skin chromatophores does not affect the sensitiveness of the larvae

TABLE 4

Photic reactions of normal Amblystoma larvae, after previous exposure in the light or in the dark

REACTIONS	CONDITION OF ADAPTATION, OF BACKGROUND, AND OF PIGMENTATION OF LARVAE											
	Dark adapted, pigmentation dark				Light adapted on black background, pigmentation dark				Light adapted on white background, pigmentation pale			
Directions of.....	+	-	=	0	+	-	=	0	+	-	=	0
Series 21.....	79	19		0	56	6	14	24				
Series 22.....	88	8		4	62	16	8	14				
Series 23.....	80	12	6	2					60	12	5	23
Series 24.....	76	13	3	8					58	14	6	22
Total number of responses.....	323	52	9	16	118	22	22	38	118	26	11	45
Per cent of responses.....	81	13	2	4	59	11	11	19	59	13	6	22

TABLE 5

Photic reactions of eyeless Amblystoma larvae after previous exposure in the light or in the dark

REACTIONS	CONDITION OF ADAPTATION, OF BACKGROUND, AND OF PIGMENTATION OF LARVAE											
	Dark adapted, pigmentation pale				Light adapted on black background, pigmentation dark				Light adapted on white background, pigmentation dark			
Directions of.....	+	-	=	0	+	-	=	0	+	-	=	0
Series 25.....	69	18	7	6	54	14	15	17				
Series 26.....	70	15	12	3	56	16	10	18				
Series 27.....	78	22	2	3					49	15	8	28
Series 28.....	68	16	6	10					52	21	11	16
Total number of responses.....	280	71	27	22	110	30	25	35	101	36	19	44
Per cent of responses.....	70	18	7	5	55	15	13	17	50	18	10	22

to light. But the reactions to light are very different according as the larvae have been adapted to light or darkness. The normal larvae which have been kept in the dark, and are, therefore, darkly pigmented are very much more sensitive than are those which have been allowed to remain in the light. When darkly pigmented larvae, which have been kept in the dark are compared with darkly pigmented larvae which have been kept in the light on a black background, it is seen that the latter are much less sensitive to light, and show a smaller percentage of positive responses, and a much larger percentage of indifferent reactions and of no reactions. The pale larvae which have been kept in the light on a white background are also much less sensitive to light than the darkly pigmented ones which have been kept in the dark, and are equally lacking in sensitiveness to those darkly pigmented larvae which have been kept in the light on a black background.

The eyeless larvae show also that those which have been kept in the dark are more sensitive to light, giving a larger percentage of positive responses than those which have been kept in the light.

In both cases, then, it may be said that dark adaptation brings about a condition of greater sensitiveness to light, irrespective of the condition of the pigmentation in the skin. The retinal pigment reacts to light probably in the way that it usually does, that is to say, when the animal is kept in the dark, the pigment migrates proximally, and when the animal is kept in the light, it migrates distally. In the dark-adapted animal then it might be assumed that, since the pigment was contracted, the retinal elements were more sensitive to light, and that the reverse might be said of the animal kept in the light. The condition of the retinal pigment probably does affect the sensitiveness of the normal larvae, although, as we have seen, the skin pigment does not have any influence on the sensitiveness of either the normal or the blinded larvae. It has been found that in some amphibians there is a connection between the nerve terminations of the eyes and those of the skin, as is to be inferred from results when the skin is illuminated. Engelmann ('85) found that changes

took place in the retinae of frogs when only the skin was exposed to light; though Fick ('90) obtained results which led him to conclude that interference with the normal respiration was the cause of these changes. Koranyi ('92) noted that illumination of the skin caused microscopic changes in the retina similar to those produced by illumination of the eye itself. In my results there is also an indication that there is such a connection between the nerve terminations of the eyes and of the skin. As has been pointed out there is a difference in the condition of the skin pigment of normal and blinded larvae under the same conditions of illumination and background. If normal larvae are placed in diffuse light on a white background they become pale (that is, the pigment in the skin chromatophores contracts); if blinded larvae are placed under the same conditions, they become dark (that is, the pigment expands). If normal larvae are placed in darkness they become dark; if blinded larvae are placed in darkness they become pale. If normal larvae are placed in diffuse light on a black background, they become dark, if blinded larvae are so placed they also become dark. In only this last case then of placing normal and blinded larvae in diffuse light on a black background are the same conditions of the skin pigment obtained. As we have seen however, in the blinded larvae, the nature of the background is of no importance, since both white and black backgrounds in diffuse light produce the same results, light and darkness being the only factors to be considered in the case of the blinded individuals. Normal larvae, on the other hand, show different conditions of pigment when placed in diffuse light on a white and on a black background. It would seem fair to conclude then that there must be some connection between the nerve terminations of the two light receptors, the eye and the skin, since in the larvae in which the eyes are left intact a different condition of the pigment of the skin is obtained from that which is found in the blinded larvae under the same conditions of illumination and background.

For the experiments which have been so far described none of the *Amblystoma* larvae were under 15 mm. in length. They all showed a sensitiveness to light, giving positive responses. At

this stage, then, all the larvae have already acquired the sensitiveness to light. It seemed worth while to attempt to find out at about what stage in the development of the larvae this sensitiveness first made its appearance. Accordingly ten young normal and ten young blinded larvae were selected, and their responses to light tested. They were placed at a distance of 25 cm. from the lamp where the light had an intensity of about 768 candle-meters. A series of readings of a thermometer placed in the water in the testing dish showed that the increase in the temperature of the water after the lamp had been burning for a period of six hours, was less than a quarter of a degree centigrade. The temperature of the room, it may be mentioned, usually rose about 1.5°C. during such a period.

These larvae when first selected were between 7 and 8 mm. long, responding to mechanical stimulation by rapid swimming movements. At this stage they were, however, insensitive to light, remaining absolutely motionless, at right angles to the rays of light. It was not until the larvae had reached a length of about 11 to 12 mm. that they showed any response.

At this stage the gills are well-developed and tufted, the balancers are fully developed, and the fore-limb buds are between 0.5 and 1 millimeter long. The larvae at this stage still lie on their sides for the most part. The ability to respond to the stimulus of light appeared rather suddenly in all. Six of the normal and five of the blinded larvae gave responses one morning, the rest were indifferent. In the afternoon of the same day they all responded to the light, giving a reaction within five minutes. The afternoon before they had all been insensitive to light. The average reaction time was longer than in the case of older larvae, being here about two hundred and twenty seconds. The larvae at this early stage all responded positively, and by means of very quick swimming movements. Sometimes a very quick movement which placed the long axis of the body in the direction of the rays was the total response. But usually they swam vigorously in the direction of the light stopping only when they came in contact with the side of the glass dish. Again, in a few cases some of them would swim vigorously for 6 or 7 cm. and then drop

passively to the bottom of the dish, always so oriented that their long axis was in the direction of the rays of light.

SUMMARY

1. Tadpoles of *R. pipiens* and of *R. sylvatica* show no response to the stimulus of light.

2. The larvae of *Amblystoma punctatum* are positively phototactic, both when normal and when deprived of their eyes.

3. The reactions of eyeless individuals are not brought about by stimulation of the central nervous system, but of the nerve terminals in the skin.

4. Normal larvae placed in light become pale, blinded larvae dark. Normal larvae placed in darkness become dark, blinded larvae pale, the conditions in the normal and blinded larvae thus being opposite.

5. The condition of the pigment in the skin chromatophores does not affect the sensitiveness of the larvae to light; but previous exposure to light, or adaptation to darkness, does, in that dark-adapted larvae are more sensitive to light than are those which have been kept in the light.

6. The youngest larvae to give responses to light of 768 candle-meters intensity were between 11. and 12 mm. long.

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EXPERIMENTS ON THE REPRODUCTION OF THE HYPOTRICHOUS INFUSORIA

II. A STUDY OF THE SO-CALLED LIFE CYCLE IN OXYTRICHA FALLAX AND PLEUOTRICHA LANCEOLATA

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SIXTEEN FIGURES (ONE PLATE)

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I. INTRODUCTION

As is well known, the early experimental work with the Infusoria gave evidence which was interpreted as supporting the view that these unicellular forms possess a definite life cycle comprising the generations between two successive conjugations, and on this account they can pass through but a certain number of generations, in the absence of conjugation, without senescence and death occurring. Calkins' work ('02) showed that

this so-called life cycle could be prolonged by 'artificial stimulation,' though he believed the cycle was real and that the stimuli used had only temporarily sustained the life of the culture in the absence of conjugation. Woodruff's work ('12 b) with *Paramaecium aurelia* has shown that, given suitable conditions, this form can be bred indefinitely and the so-called life cycle entirely eliminated. In his work with *Paramaecium caudatum*, the same investigator has shown that what appeared to be a typical life cycle in this organism was merely the dying out of the race due to unfavorable culture conditions. In Part I of these studies the writer has shown that fundamental changes can be brought about in the life history of the descendants from the same original animal by keeping the progeny on different culture media.

It appears, then, that the environment plays an important part in determining the length of the so-called life cycle and the character of the life history in the Infusoria. Inasmuch as the experimental work on the hypotrichous Infusoria has given evidence that these animals have a definite life cycle, it was decided to re-investigate the problem and endeavor to discover culture conditions in which these forms will live indefinitely without conjugation or artificial stimulation and thus eliminate the so-called life cycle for these hypotrichida as has been done by Woodruff with *Paramaecium*. Accordingly, the experiments recorded in this paper were undertaken with the view of finding out the life history of the organisms studied when kept on different media and under varying culture conditions. In order to accomplish this, a number of cultures containing descendants of the same original individual were run at the same time under various culture conditions and consequently the life history of these cultures gives direct evidence on the effect of these varying conditions on the 'same protoplasm.' It was hoped in beginning the work that the conditions encountered, in some culture or cultures of the organisms studied, would be such as to permit the indefinite existence of the animals, and that the 'life cycle' might be eliminated in these forms. Also, if 'sister cells' kept under other culture conditions, which to begin with appeared to be entirely suitable, died out after passing through what ap-

peared to be a typical 'life cycle,' it would then be evident that they had not died through the ending of a definite life cycle, due to an inherent condition of senescence, but because the culture conditions had not been entirely suitable, inasmuch as 'sister cells' under other culture conditions were able to live indefinitely. In one of the two species employed in this work, *Pleurotricha lanceolata*, these expectations have been realized. The experimental work embraces a study of the following points:

1. The life history of *Oxytricha fallax* when bred in beef extract, hay infusion and 'varied environment' media under the conditions of daily isolation cultures.

2. The life history of the same organism when bred in a beef extract medium in mass cultures in test tubes.

3. The life history of *Pleurotricha lanceolata* when bred in beef extract and hay infusion media under the conditions of daily isolation cultures.

4. A study of a mass culture of *Pleurotricha lanceolata* kept on a hay infusion medium in test tubes.

These experiments have been carried on during a period of two years in the following laboratories: Sheffield Biological Laboratory and Osborn Zoölogical Laboratory of Yale University, Biological Laboratory of Central College, Pella, Iowa, and the Marine Biological Laboratory, Woods Hole, Massachusetts.

The writer desires to acknowledge his great obligation to Professor Lorande L. Woodruff for suggesting the problem and for the splendid assistance given during the course of the work, and also to Professor Alexander Petrunkevitch for assistance in making the microphotographs.

II. METHODS

In Part I of these studies is given a full description of the methods used in carrying on the daily isolation cultures, the making of the beef extract, hay infusion and 'varied environment' media, the preparation of permanent specimens, and so forth. Inasmuch as all of these methods have been used in the present paper without modification, the reader is referred to the previous paper for these details.

III. HISTORY OF THE CULTURES

1. *Oxytricha fallax*

The culture work with this hypotrichous infusorian was begun on October 19, 1911, by the isolation of a single specimen from a laboratory hay infusion. The individual was placed on a depression slide and some of the regular beef extract medium added. The progeny from this individual furnished the material for all the cultures of *Oxytricha fallax* mentioned in this paper. Four different cultures were under observation and each of these is described in detail in the following sections.

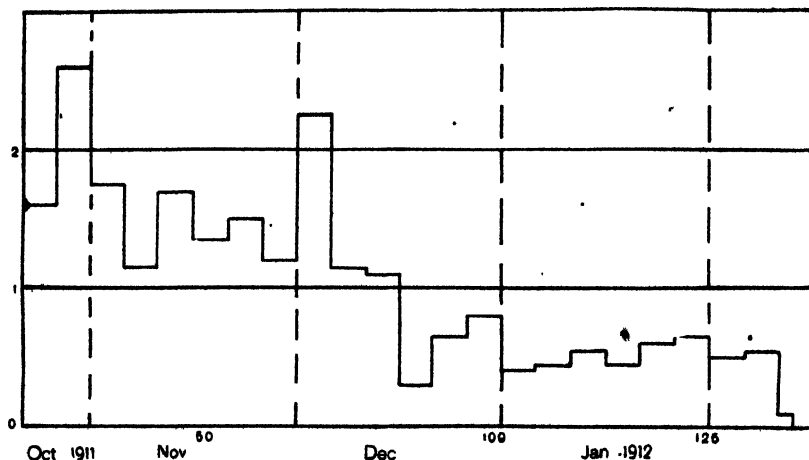


Diagram 1 Graph giving the life history of *Oxytricha fallax*, Culture Ob, showing the average daily rate of division of the four lines of the culture, again averaged for five-day periods. Beef extract culture medium.

a. *Culture Ob*. This daily isolation culture, bred on the bee medium, was started October 19, 1911, and continued until February 10, 1912, a period of 115 days, during which time it passed through 131 generations. Diagram 1 shows graphically the life history of this culture. With the exception of two five-day periods, the division rate was at all times below two divisions per day, and during the last two months the rate was never as

high as one division per day when averaged for five-day periods. Although the division rate was low, the animals appeared to be normal and healthy during the first three months of the culture, but about this time abnormal individuals began to appear and subsequently the culture died.

b. Culture Oh. This daily isolation culture, bred on the hay infusion medium, was started October 27, 1911, by isolating an individual from each of the four lines of the Ob culture, and was carried until February 8, 1912, a period of 105 days, during which time it passed through 159 generations. Diagram 2 shows graphically the life history. A study of the graph and a comparison

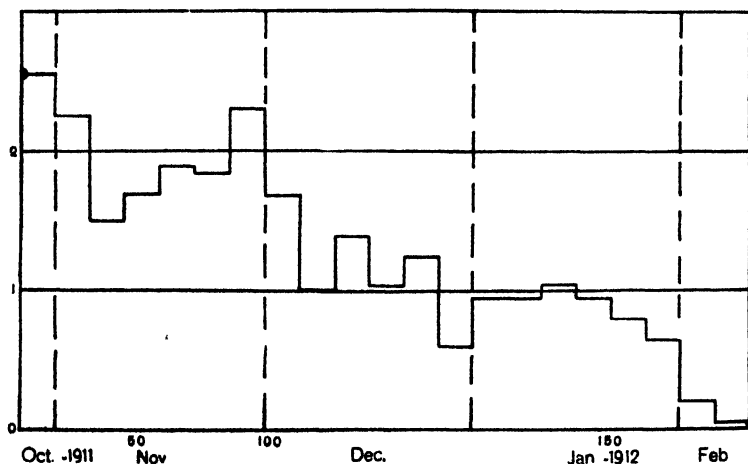


Diagram 2 Graph giving the life history of *Oxytricha fallax*, Culture Oh, showing the average daily rate of division of the four lines of the culture, again averaged for five-day periods. Hay infusion culture medium.

with diagram 1 shows that the life histories of these two cultures were much the same. This culture maintained a slightly higher division rate than did the Ob culture but died a few days sooner.

c. Culture Ove. This daily isolation culture, bred on the 'varied environment' medium, was started October 27, 1911, by isolating an individual from each of the four lines of the Ob

culture, and was carried until January 14, 1912, a period of 80 days, during which time it passed through 150 generations. Diagram 3 shows graphically the life history. Although this culture did not live so long as either of the other cultures it maintained a considerably higher rate of division. However, the decline which began about the middle of December continued quite rapidly and steadily and the culture died, as noted above.

It is observed, then, that, in all three of the daily isolation cultures of *Oxytricha fallax*, there is a general decline in the

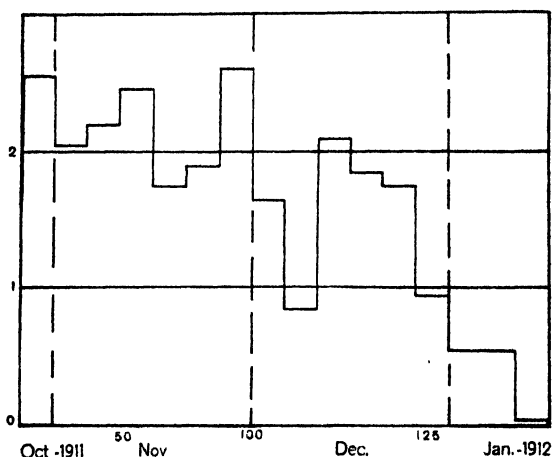


Diagram 3 Graph giving the life history of *Oxytricha fallax*, Culture Ove, showing the average daily rate of division of the four lines of the culture, again averaged for five-day periods. 'Varied environment' culture medium.

division rate almost from the time the cultures were first started. Of the three media used it appears that the 'varied environment' medium was to a slight degree less suitable for this animal than either the beef or the hay media. The animals of the Ove culture died out almost a month previous to those of the other two cultures. The rate of division was lower in the Ob culture than in either of the others but on the other hand this culture lived the longest. The highest number of generations was attained by the Oh culture. In brief, while some minor differ-

ences were evident in the life history of these three cultures in the way of division rate and length of time which they lived, they all showed what might be termed typical 'life cycles' ending with the death of the organisms.

d. Culture Obt. A few days after the starting of the Ob culture, October 22, 1911, the animals from one of the stock slides were placed in a test tube containing some of the beef extract medium. This proved to be a favorable environment for the animals and the culture was continued by the following methods: The sterile beef extract medium is kept in test tubes, about 25 cc. in each. Into such a tube is introduced a little culture medium containing some of the animals. The animals thus introduced multiply very rapidly, if the conditions are favorable, and in about four or five days the maximum number is usually present. After this time the number of animals present gradually decreases, due chiefly, it is supposed, to a lack of sufficient food supply. When about the maximum number of animals is present, a little of the medium containing some of them is introduced into a fresh tube of the beef extract medium and thus the culture is continued. The bacteria which are introduced at the time of inoculation also multiply very rapidly in the fresh medium and furnish the food supply for the animals of the culture. The test tubes containing the animals are examined with great care daily by means of a Zeiss binocular microscope. By the use of such an instrument it is quite easy to examine the individuals contained in a small test tube very thoroughly and to be certain that conjugation is not occurring.

This mass culture in the test tubes was continued on the beef medium from October 22, 1911, until May 15, 1912. It was noted at this time that the animals in the culture were not multiplying as rapidly as usual and it was thought that a change of medium might be beneficial. Accordingly a large number of media of different kinds and varying strengths were made up from materials gathered from ponds and also from hay and grasses taken from different localities. These infusions were boiled to prevent contamination of the culture by foreign species

of Infusoria which might be present, and placed in test tubes. On most of these new infusions the animals thrived very well and were present in large numbers in the test tubes a few days later. Some of the animals were also kept as before on the beef medium in the test tubes but these all died by May 20. The other animals which had been transferred to the new media from the beef medium showed for a time a great improvement and appeared to have recovered their normal division rate but, about three weeks later, they again showed a low division rate and other signs of degeneration and this time the culture died out (June 10, 1912) after having been carried 231 days or almost eight months. It has been shown, as mentioned above, that the maximum time that these animals lived, in any of the daily isolation cultures, was 112 days. We have, then, under the conditions in the mass culture more than a doubling of the length of the time of the so-called life cycle as found in the daily isolation cultures.

To summarize briefly the culture work with this race of *Oxytricha fallax*: Three daily isolation cultures and one mass culture in test tubes were carried, namely, Culture Ob, a daily isolation culture which attained 131 generations in 115 days on a beef extract medium; Culture Oh, a daily isolation culture which attained 159 generations in 105 days on a hay infusion medium; Culture Ove, a daily isolation culture which attained 150 generations in 80 days on a 'varied environment' medium; Culture Obtt, a mass culture kept in test tubes which lived 231 days on the beef extract medium. It is obvious from the experiments with this race of *Oxytricha fallax* that, although it was impossible to prolong the life of the race indefinitely by any of the media employed, nevertheless it was possible, by the use of the test tube method to greatly extend the so-called life cycle. This result supplies additional evidence that the length of the 'life cycle' and the character of the culture methods are intimately related.

2. *Pleurotricha lanceolata*

The culture work with this hypotrichous infusorian was begun on January 6, 1912, by the isolation of a single specimen from a laboratory hay infusion. This individual was placed on a depression slide and some of the regular hay infusion added. The species proved to be one that was well adapted for experimental work of this kind, and the progeny from this individual furnished

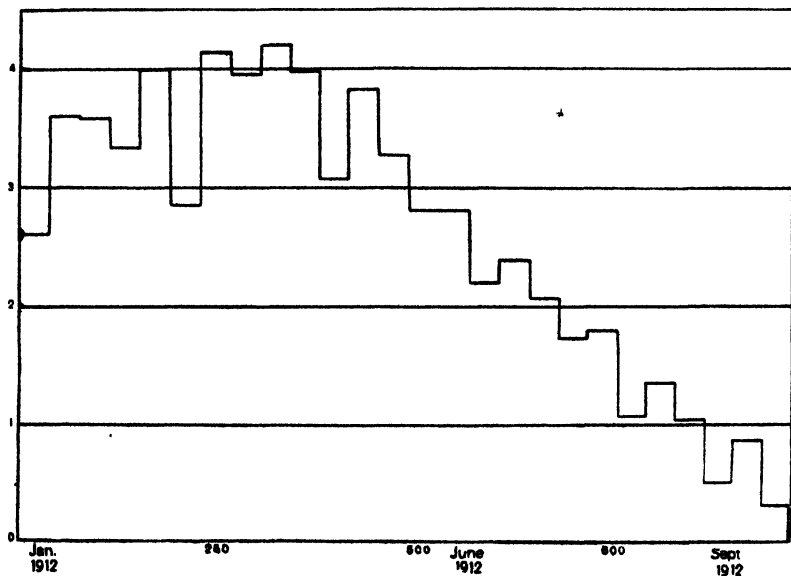


Diagram 4 Graph giving the life history of *Pleurotricha lanceolata*, Culture Ph, showing the average daily rate of division of the four lines of the culture, again averaged for ten-day periods. Hay infusion culture medium.

the material for all the cultures of *Pleurotricha lanceolata* mentioned in this paper. Three different cultures were under observation and each of these is described in detail in the following sections.

a. Culture Ph. This daily isolation culture, kept on the hay medium, was started January 9, 1912, and continued until September 25, 1912, a period of 259 days, during which time it

passed through 656 generations. Diagram 4 shows graphically the life history of this culture. At the time the culture was started the animals were dividing at an average rate of 2.5 divisions in twenty-four hours. This rate gradually increased for a time as shown in the graph until the culture reached the remarkably high rate of four divisions per day. The records show that an average rate of about four divisions per day was maintained for forty days. However, even though the division rate was very high and the animals of the culture gave every evidence, so far as could be determined, of being in an environment which was suited to their needs, a decline in the rate began at the end of the forty-day period mentioned above and this decline steadily continued and finally resulted in the death of the culture (September 25, 1912).

b. Culture Pb. This daily isolation culture kept on the beef medium, was started January 9, 1912, and carried until May 1, 1913, a period of 478 days, during which time the culture passed through 943 generations. Diagram 5 shows graphically the life history of this culture. As will be noted in the graph, a division rate averaging about 2.5 divisions in twenty-four hours was maintained for the first twenty periods (200 days). The decline which began at this point continued very slowly but steadily, the rate finally falling below two divisions in twenty-four hours and during the last two months of the life of the culture the rate was never as high as one division per day when averaged for a ten-day period. The culture finally died out at the 943d generation on May 1, 1913.

In both of these daily isolation cultures of *Pleurotricha lanceolata*, a study of the graphs reveals what could be termed typical 'life cycles.' When once the decline began with few exceptions it steadily continued and the maximum of each succeeding rhythm generally was lower than the maximum of the preceding one.

c. Culture Phtt. This mass culture kept on a hay infusion medium in test tubes was started January 9, 1912, at the same time that the two daily isolation cultures (Ph and Pb) were started and has been carried continuously since then up to the present

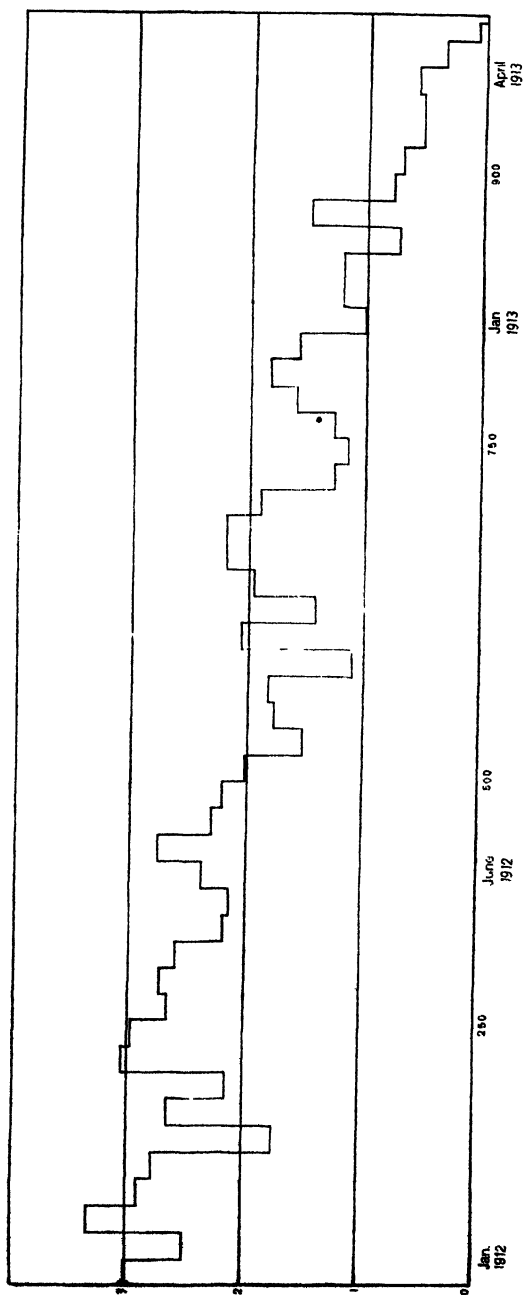


Diagram 5 Graph giving the life history of *Pleurotricha lanceolata*, Culture P1, showing the average daily rate of division of the four lines of the culture, again averaged for ten-day periods. Beef extract culture medium.

time (November 20, 1913), a period of more than twenty-two months, under these same conditions.

The method of carrying a test tube culture has already been described above (see Culture Obtt, p. 217) and this same method has been used in this case, except that the medium used has been hay infusion instead of the beef medium.

At any time during the period that the culture has been under observation it has been possible to secure almost any number of animals simply by inoculating a suitable medium with a few of them from the regular test tube culture. Great numbers of the animals obtained in this way have been used in class work, for the study of both living and prepared material, during the course of the experiments, and no abnormal specimens have ever been found. In order to test the division rate of the animals from this culture after they had been subjected to the culture conditions described above for almost twenty months, a daily isolation culture on the hay infusion medium was started on July 8, 1913, and continued for a period of 35 days. In starting it, one individual was taken from this test tube culture and placed on a depression slide. During the night this individual divided twice, forming four animals, and these were isolated and placed on depression slides to start the four lines of the daily isolation culture. During the 35 days that this culture was under observation, the animals maintained a division rate of almost exactly 2.5 divisions per day. It is evident from this experiment that the animals of this culture are capable of maintaining as high a division rate now as when the culture was first started.

The animals of this test tube culture have given at all times during the more than 22 months that they have been under observation every evidence of being in an environment which is entirely suitable for them and *it is believed that these animals can be bred indefinitely under these conditions without conjugation or artificial stimulation of any kind.*

To summarize briefly the culture work with this race of *Pleurotricha lanceolata*: Two daily isolation cultures and one mass culture in test tubes were carried, namely: Culture Ph, a daily isolation culture which attained 656 generations in a period of 259

days on a hay infusion medium; Culture Pb, a daily isolation culture which attained 943 generations in a period of 478 days on a beef extract medium; Culture Phtt, a mass culture kept in test tubes on the hay infusion medium, which is still thriving at the present time (November 20, 1913) more than 22 months after it was first started and which gives every evidence of being able to live indefinitely under these conditions without conjugation or artificial stimulation.

IV. PREPARED MATERIAL

The study of the prepared material of *Oxytricha fallax* and *Pleurotricha lanceolata*, taken from the cultures which died out, reveals the fact that unsuitable culture conditions may bring about great nuclear and cytoplasmic changes in these organisms.

1. *Oxytricha fallax*

Figures 1 to 6 are from material taken from the *Oxytricha fallax* cultures. The animals preserved at the beginning of the work showed a normal morphological condition, as is seen, for example, in figure 1. Among the individuals preserved a month or so later occasionally an abnormal individual was found. An example of this is shown in figure 2. The cytoplasm of this animal still appears to be in a practically normal morphological condition but in the nuclear structure is to be seen evidence of degenerative changes. As the cultures became still older a very much greater proportion of atypical individuals were found among the specimens preserved and in many cases the appearance of the animals while still alive showed that they were not normal. Shortly before the death of the daily isolation cultures practically every animal that was preserved showed a degenerate condition. In figures 3 and 4, which are from specimens preserved later in the work, are shown other specimens of degenerate animals. Figures 1. to 4 were all taken from specimens preserved from the daily isolation cultures.

The animals mentioned above apparently do not differ from those obtained by former investigators along this line and by

whom the degeneration was thought to be due to an inherent condition of senescence. In these experiments, it can be said that such was not the case, for 'sister cells' which were kept under other conditions in the mass test tube culture were, as far as could be told both by careful observation of the living animals and a thorough study of the prepared specimens, normal at the time when, and for some time after, the daily isolation cultures had died out. In figure 5 is shown a typical example of the animals present in the test tube culture at this time. This specimen was preserved February 15, which was several days after the last of the daily isolation cultures had died. Figure 6 is a specimen preserved from the test tube culture May 18 and it shows an atypical condition of the nucleus and cytoplasm. Before this time specimens preserved from this culture gave every evidence of being in as normal condition as is shown in figure 5. After this time, however, the number of abnormal animals in the test tube culture rapidly increased, due, it is believed, as in the daily isolation cultures of this organism, to the cumulative effects of an environment not entirely adapted to the organism studied, and subsequently (June 10, 1912) this culture died.

2. *Pleurotricha lanceolata*

Figures 7 to 11 are from *Pleurotricha lanceolata* cultures. In figure 7 is shown a typical normal individual. This specimen was taken from the Ph culture soon after it was started. Figure 8 shows an animal taken from the same culture several months later shortly before it died out. Another atypical specimen is seen in figure 9 which was taken from the Pb culture at about the same time. As in the *Oxytricha fallax* cultures so also here in the *Pleurotricha lanceolata* cultures the changes which appeared in the animals in the cultures that died out is shown not to be due to 'senile degeneration' inasmuch as 'sister cells' which were kept under other culture conditions do not show simultaneous degenerative changes. Figure 10 is a typical normal specimen preserved from the Ph_{tt} culture some time after

the death of the Ph culture, and in figure 11 is shown another normal individual from the same culture preserved October 20, 1913, *about 13 months after the death of the Ph culture and 6 months after the death of the Pb culture.* As has been noted above, *no abnormal specimens have ever been found in the Phll culture during its entire history, covering, thus far, a period of over 22 months.*

V. DISCUSSION AND CONCLUSIONS

A number of investigators have noted the fact, as shown in these experiments, that closely related species of the Infusoria, or even different races of the same species, vary greatly in regard to culture conditions that are adapted to them. Jennings and Hargitt ('10), working on a number of different races of paramaecia, found that culture conditions which were apparently ideal for a certain race of *Paramecium* might not be at all suitable for another race. Woodruff ('11) found that culture conditions which were entirely suitable for his *Paramecium aurelia* cultures and on which they had thrived for years were not entirely adapted for a race of *Paramecium caudatum*. The experiments of Woodruff ('12), in his researches on the protozoan fauna of hay infusions, indicate that the animals are adapted for only certain conditions of the medium, and when these conditions are attained, they are present in maximum numbers, and as the conditions in the infusion change they disappear and other forms become dominant which are adapted to the new conditions.

In Part I of these studies, the writer ('12) was able to show that it is possible to change greatly the life history of *Stylonichia pustulata* by employing different kinds of culture media. In a culture bred on beef extract medium conjugation occurred, whereas, in another culture of descendants from the same original animal but kept on a hay infusion medium, conjugation did not occur at any time. The animals in the beef culture died at the 403d generation while those on the hay medium attained 572 generations. The experiment was repeated by starting another culture on the beef extract medium with animals from the hay culture after the first beef culture had died, and again conjuga-

tion occurred in this second beef culture. Inasmuch as the only difference in the treatment of the two cultures lay in the media used, there can be no doubt that the fundamental changes in the life histories of the hay and beef cultures were induced by the culture media. To quote from the former paper (p. 71):

Inasmuch as conjugation never occurred at any time in the hay culture, even though in both the length of time it was kept and the number of generations through which it passed it exceeded either of the cultures kept on the beef medium, there is conclusive evidence that neither the age of the organisms nor the number of generations through which they passed were potent in inducing conjugation in these cultures. *The determining feature was the medium used and the results here recorded give definite evidence that the 'same protoplasm' under the influence of different culture media may show fundamental differences in its life history.*

Calkins ('13), in some recent experiments on *Paramecium*, isolated the four cells resulting from the first two divisions of an ex-conjugant and, later, when each of these four cells had divided three times, forming eight cells, or 32 cells in all, he again isolated them, forming four 'quadrants' of eight lines each which were continued as pure lines. During the time that the animals have been under observation (about seven months) conjugation has been found to occur in only one of the 'quadrants.' Calkins concludes from these results, but more particularly from the fact that no conjugation has occurred in Woodruff's *paramecium* cultures during their long period under observation,¹ that "some descendants of an ex-conjugant are potential germ cells. others are not" or, in other words, that in *paramecia* there are conjugating and non-conjugating races, due to an inherent difference in the four cells derived from a single ex-conjugant. However, Calkins in the preceding volume of this journal presents the results in a complete form. These show that conjugants have now been found in *all* the 'quadrants' of the original *J* series. To quote from page 448: "The April test brought a surprise" in that animals from 'quadrants' *B*, *C* and *D* all gave some conjugating pairs, as they also did in a second test later

¹For later results see article by Dr. Woodruff in this number.

in April, so that "all four quadrants are now represented in the conjugating lines." Inasmuch, then, as all the 'quadrants' are now shown by these later results to possess the power of conjugation, the results of the experiments do not seem to be such as to prove that there are conjugating and non-conjugating races present in these cultures. The explanation as to why some of the 'quadrants' conjugate more freely than others may lie in some external condition or conditions of the environment rather than in any inherent difference in the animals of the various 'quadrants,' since, as noted above, my work with *Stylonychia pustulata* has shown that a 'conjugating race' and a 'non-conjugating race,' using Calkins' terms, may be obtained from the descendants of a single individual not an ex-conjugant by the subjection of the different lines to various culture conditions.

In the experiments recorded in the present paper it has been shown that *Oxytricha fallax* under certain culture conditions, which at the beginning of the work appeared to be entirely suitable, lived about three and one-half months, but that under other culture conditions they lived nearly eight months, or more than twice as long. In *Pleurotricha lanceolata*, also, the ability of different culture conditions to vary the life history of the organism has been clearly shown in that one of the cultures lived 259 days, another lived 478 days, and another gives every promise, after over 22 months of existence, of having culture conditions which are entirely suitable and in which it can live indefinitely without conjugation or artificial stimulation.²

The graphs showing the division rate of all the daily isolation cultures, of both the organisms studied, exhibit what may be

² I cannot agree with the position of Calkins and Gregory ('13) which appears to be essentially that in order to 'prove' the 'immortality' of the Protozoa they must be bred through eternity. The generally accepted work of Woodruff with a race of *Paramecium aurelia* has established beyond the question of a doubt, I believe, that in this form at least an indefinite and unlimited multiplication without conjugation or artificial stimulation may be had, provided suitable culture conditions are supplied. The experiments in these studies show that the same holds true for at least one of the hypotrichous Infusoria and also that the so-called life cycle may be greatly and variously modified, depending upon the conditions in which the animals are bred.

termed 'typical life cycle' curves. Also the study of the prepared material, from the cultures of both species which died out, reveals morphological and nuclear changes which can be interpreted as being due to 'senile degeneration.' However, since, as has been noted above, 'sister cells' from the test tube cultures of *Oxytricha fallax*, preserved at the same time or even later than cells in a degenerate condition taken from the daily isolation cultures and which should therefore show at least as great a degeneration, reveal a normal nuclear and cytoplasmic condition and, also, because of the fact that in *Pleurotricha lanceolata* no degenerate or abnormal individuals have ever been found in the test tube culture during the entire period that it has been under observation, it is evident that the atypical conditions that were present in the cells of the cultures which died cannot be attributed to an inherent condition of senescence. The results obtained clearly indicate that the degenerative changes which occurred and the subsequent dying out of the cultures resulted not from a condition of 'senile degeneration' in the animals, but from the fact that the culture conditions supplied in these cases were not entirely suitable, and consequently, it is believed, that the length of the so-called life cycle in these hypotrichous infusorian forms is not a question of an inherent ability to attain only a certain number of generations, but it is dependent upon the degree to which the culture conditions are favorable. *If the conditions supplied are entirely adapted to the needs of the organism, as in the test tube culture of Pleurotricha lanceolata, it seems evident that there will be no so-called life cycle and the organism will continue to multiply as long as these conditions are maintained.*

When the results of some of the earlier investigators which support the view that the Infusoria possess a definite life cycle ending at a certain number of generations are considered, it appears that these results have been due to the cumulative effects of culture conditions which were to some degree unsuitable to the organisms under observation and which, therefore, were not adapted to their indefinite existence.

The question of artificial stimulation as a means of prolonging the so-called life cycle of various protozoan forms should also

be mentioned. Calkins ('02), for example, has shown in his work with *Paramecium caudatum* that, at times, a decided change of medium may result in a 'rejuvenation' and the animals which before had been dividing very slowly and showing other signs of degeneration, will take on a new lease of life and appear normal for a time. This 'artificial rejuvenation' could be accomplished a number of times but finally the organism reached a stage where no 'rejuvenation' resulted from any of the methods tried and the animals of the culture died out. Woodruff ('05) also found in his work with one of his cultures of *Oxytricha fallax* that these animals were 'artificially rejuvenated' by the use of a beef extract medium. At about the 300th generation, after the culture had been under observation for about ten months, the animals of the culture were dividing very slowly and it seemed that they would all die, when they were 'rejuvenated' by the use of the beef extract and finally reached the 860th generation, almost a year later, before the culture died out. At the time it was believed that the degeneration, which appeared in the animals of the cultures under observation, was due to an inherent condition of senescence, but it would appear, from the more recent researches, that the degeneration resulted from the cumulative effects of culture media or culture conditions which were not entirely adapted to the organisms in question. Woodruff in a recent paper ('13) holds this view with regard to his culture of *Oxytricha fallax* and bases his conclusion upon the results of his work with *Paramecium aurelia*. To quote (p. 8):

Therefore in the light of studies during the past decade, I would interpret the cycle of my *Oxytricha* A culture as being the result of the fact that the race of *Oxytricha fallax* which was employed was not adapted to live indefinitely 'when continuously subjected to the same environment' of hay infusion ('05, p. 627). I believe that if an entirely suitable environment had been secured this culture would have given evidence of unlimited power of reproduction by division without conjugation as my present *P. aurelia* race has done. •

In the cases of 'artificial rejuvenation,' it seems evident, then, that the change of medium brought about a temporary relief

from unsuitable culture conditions but since none of the media supplied happened to be entirely suited to the needs of the organism, death invariably resulted after a time.

From the results of these experimental studies mentioned above it is believed that only one conclusion can be drawn and that is that *the length of the so-called life cycle in the hypotrichous Infusoria, as in Paramecium, is not a question of senescence or, in other words, a tendency of the protoplasm to grow old, but it is dependent upon whether or not the organisms are supplied with an environment which is adapted to their particular requirements.* Given a species which is adapted to the culture conditions in which it is bred there is reason for believing that it can be bred indefinitely without the necessity of conjugation or of artificial stimulation. If the culture conditions supplied are unfavorable to the organism, marked nuclear and cytoplasmic degenerative changes occur and the death of the culture inevitably results, sooner or later, depending upon the degree to which the conditions are unfavorable, the natural resistance of the species, and its ability to adapt itself to the new conditions.

VI. GENERAL SUMMARY

1. The object of these experiments was (1) to determine if culture conditions could be supplied to the hypotrichous Infusoria which would eliminate the so-called life cycle in these forms, and (2) to observe the effect, upon the life history of the organisms studied, of the various culture conditions supplied.

2. Three media were used in these experiments, namely, a 'constant' medium consisting of a 0.025 per cent solution of Liebig's extract of meat, a hay infusion medium, and a 'varied environment' medium. Both daily isolation cultures and mass cultures kept in test tubes were employed in this work.

3. Four cultures of *Oxytricha fallax* have been under observation, all of which were started from descendants of an original individual isolated from a laboratory hay infusion, October 19, 1911. Culture Ob was started October 19, 1911, and was carried until February 10, 1912, on a beef extract medium, when it died

out at the 131st generation. Culture Oh was started October 27, 1911, by isolating an individual from the Ob culture, and it was carried until February 1, 1912, on a hay infusion medium, when it died out at the 159th generation. Culture Ove was started October 27, 1911, by the isolation of an individual from the Ob culture and it was carried until January 14, 1912, on a 'varied environment' medium, when it died out at the 150th generation. These three were all daily isolation cultures. Culture Obtt, a mass culture, carried on a beef extract medium and kept in test tubes, was started October 22, 1911, by the isolation of an individual from the Ob culture and was carried until May 15, 1913, when it died out. Graphs showing the life history of each of the daily isolation cultures have been plotted by averaging the divisions in the four lines of each culture for five-day periods.

4. Three cultures of *Pleurotricha lanceolata* have been under observation, all of which were started from descendants of an original individual isolated from a laboratory hay infusion January 6, 1912. Culture Ph was started January 9, 1912, and was carried on a hay infusion medium until September 25, 1912, when it died out at the 656th generation. Culture Pb was started January 9, 1912, and was carried until May 1, 1913, on a beef extract medium, when it died out at the 943d generation. Both of these were daily isolation cultures. Culture Phtt, a mass culture kept in test tubes on a hay infusion medium, was started January 9, 1912, and has, so far, been carried over 22 months and gives every evidence of being able to live indefinitely. Graphs showing the life history of each of the daily isolation cultures have been plotted by averaging the divisions in the four lines of each culture for ten-day periods.

5. The study of the graphs in all the daily isolation cultures of both species reveals what might be termed typical 'life cycles' in these organisms.

6. The study of the prepared material shows that, although the animals at the beginning of the work gave every evidence of a normal morphological condition, previous to the death of a culture atypical and degenerate individuals appear and these

are similar to degenerate animals found by former investigators, by whom they were thought to be due to an inherent condition of senescence in the animals.

7. In these experiments the results indicate that the dying out of some of the cultures was due, not to a condition of inherent senescence, but to the fact that the culture conditions supplied were not entirely favorable to the indefinite existence of the organisms under observation, since in *Oxytricha fallax* 'sister cells' bred in a mass test tube culture lived more than twice as long as those bred in daily isolation cultures, and in *Pleurotricha lanceolata* culture conditions have been found in which this organism *will apparently live indefinitely without conjugation or artificial stimulation.*

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PLATE 1

EXPLANATION OF FIGURES

These photographs were taken from permanent preparations stained with picrocarmin. The same magnification (600 diameters) was used in all cases and, therefore, the relative sizes represent absolute differences in the size of the different individuals.

Oxytricha fallax

1 A typical normal individual taken from the Oh culture at the beginning of the work. One of the micronuclei has divided prior to cell division.

2 to 4 In these figures are to be seen types of the abnormal animals which appeared later in the history of the cultures. Figure 2 was taken from the Ove culture, December 12, 1911, at the 110th generation. Figure 3 was taken from the Oh culture January 27, 1912, at the 158th generation. Figure 4 was taken from the Ob culture January 27, 1912, at the 121st generation.

5 A typical normal individual taken from the Obt culture on February 15, 1912, several days after the death of the last of the daily isolation cultures.

6 An atypical individual taken from the Obt culture on May 18, 1912, about two weeks before this culture died out.

Pleurotricha lanceolata

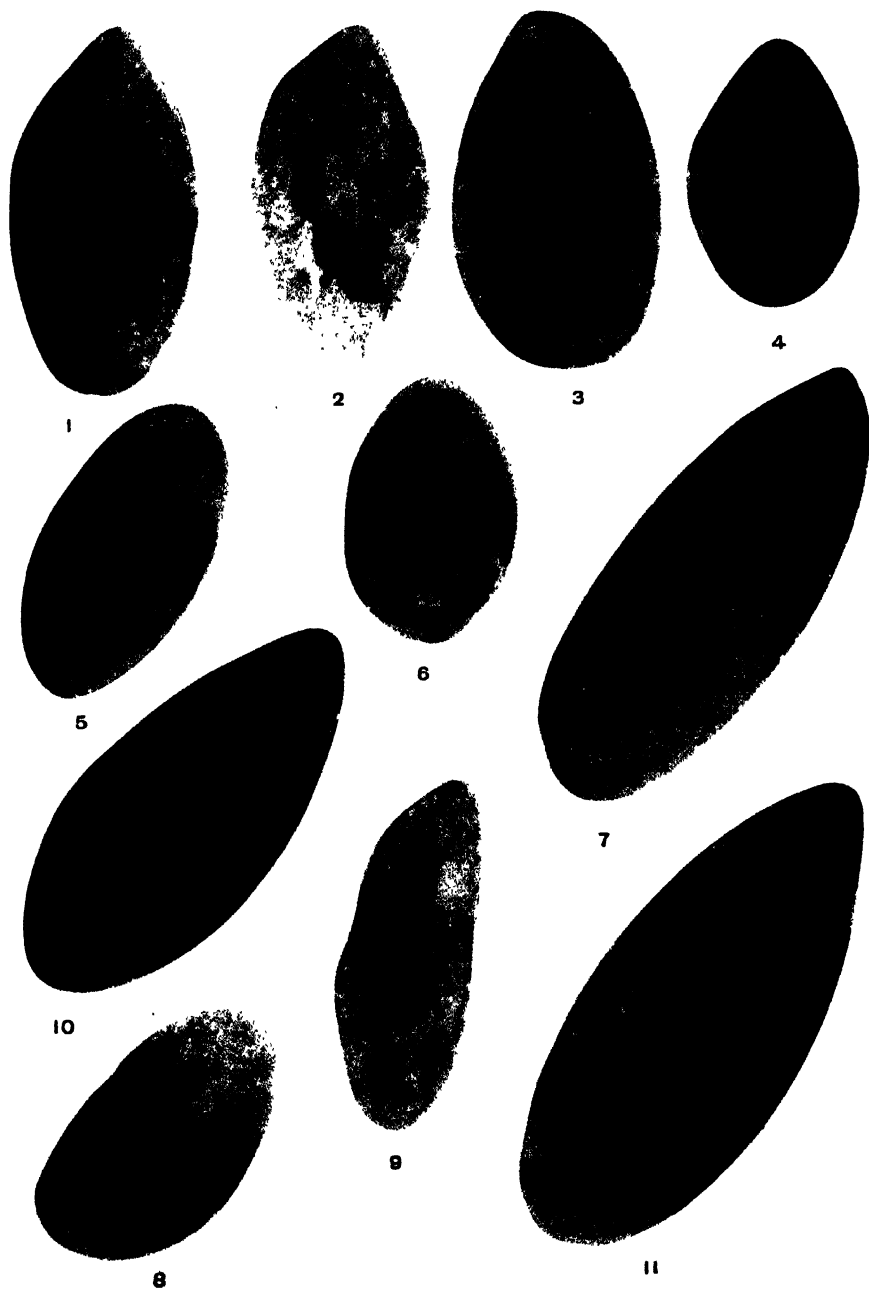
7 A typical normal individual taken from the Ph culture at the beginning of the work.

8 An atypical individual taken from the Ph culture August 28, 1912, at the 637th generation. This culture died out four weeks later.

9 An atypical individual taken from the Pb culture August 28, 1912, at the 597th generation.

10 A normal individual taken from the Phtt culture September 7, 1912.

11 A normal individual taken from the Phtt culture October 20, 1913.



SO-CALLED CONJUGATING AND NON-CONJUGATING RACES OF PARAMAECIUM

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ONE FIGURE

An interesting characteristic of the pedigreed race of *Paramecium aurelia*,¹ which I have had under daily observation for the past six years and eight months, has been the apparent abeyance of the tendency to conjugate during the first seventy-nine months of its existence. Although the possibility of conjugation has been prevented in the main lines of the race, a large series of experiments have been made from time to time to induce conjugation in mass cultures started with individuals left over from the daily isolations from the main lines. These experiments apparently failed to afford just the proper environmental conditions to initiate conjugation² for not a single pair had been observed before the experiment to be described below.

On December 1, 1913, a large jarful of water and decaying plants from an old laboratory amoeba culture was thoroughly boiled for half-an-hour and a part of this material was then put into a large, sterilized stender dish with ground-on cover, while the rest was left in the container in which it was boiled. The following day the contents of the stender dish was inoculated with a considerable number of paramaecia left over from the isolations that day (4102d generation).³ At the end of five days there was a heavy pure growth of paramaecia in the stender dish but no protozoa of any kind in the uninoculated culture material.

¹ For details of the race, cf. L. L. Woodruff: *Archiv für Protistenkunde*, Bd. 21, 1911; *Biochemical Bulletin*, vol. 1, 1912.

² E.g., Baitsell (*Jour. Exp. Zool.*, vol. 13, 1912, p. 73) from his experiments on the life history of *Stylonychia* shows "that conjugation is induced by external conditions affecting the organism."

³ Cf. figure 1.

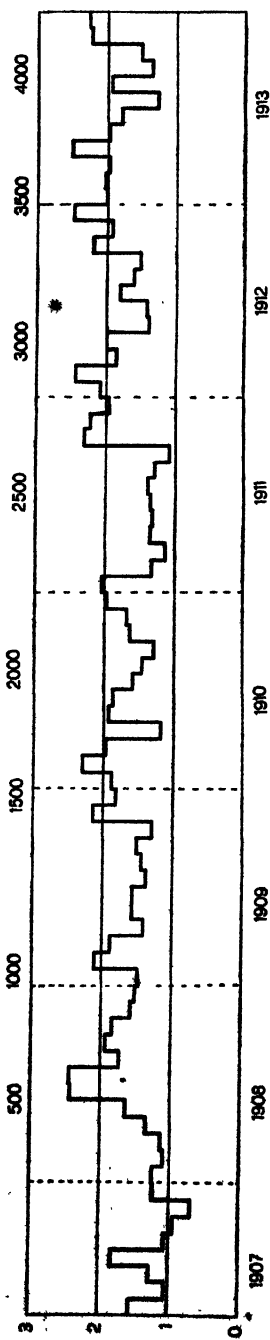


Fig. 1 Graph showing the rate of reproduction of the pedigreed race (I) of *Paramaecium aurelia* from start on May 1, 1907, to the present time, January 1, 1914, at the 4167th generation. The ordinates represent the average daily rate of division of the four lines of the culture again averaged for each month of its life to date. The vertical broken lines indicate the limits of the calendar years. The figures 500, 1000, etc., represent generations attained at the various periods.

Eight depression slides were each filled with about five drops of this culture of *Paramecium* and placed in a moist chamber at room temperature. The following morning (December 7, 1913) a couple of conjugating pairs were discovered on each slide. At this time material from the stender dish was again distributed on depression slides and the next day again a couple of pairs were isolated from several of these slides. About twenty pairs were thus secured, some of which have been preserved at various stages for cytological study and others as exconjugants initiated lines which are now being bred by the same pedigreed culture methods employed for the race from which they were originally derived. Details of this work will be presented in a later paper.

During the more than six and one-half years of the life of this race a considerable amount of evidence has been accumulated by different observers tending to show that the conditions determining conjugation vary greatly in different races and in different lines within the same race of *Paramecium aurelia* and *Paramecium caudatum*. Jennings⁴ writes: "Some races conjugate frequently, and under conditions readily supplied in experimentation. Others, under the same conditions, conjugate very rarely or not at all." Calkins⁵ finds that "Some lines will conjugate whenever the conditions favorable for conjugation are prepared; other lines have never conjugated under such conditions."

These variations in the tendency to conjugate which are exhibited by pure races and lines of *Paramecium* apparently have led Calkins to believe that "The traditional view that each *Paramecium* is a potential germ cell is not true,"⁶ and that herein lies the clue to the directly opposite results derived from his races and from mine.⁷

⁴ H. S. Jennings, Jour. Exp. Zool., vol. 9, 1910, p. 298.

⁵ G. N. Calkins and L. H. Gregory, Jour. Exp. Zool., vol. 15, 1913, p. 509.

⁶ G. N. Calkins, Proc. Soc. for Exp. Biol. and Med., vol. 10, 1913, p. 67.

⁷ Calkins (loc. cit.): "The life history of conjugating lines has shown that if conjugation is prevented, the race dies out." Woodruff (loc. cit.): "I believe this culture shows clearly that *Paramecium aurelia* when subjected to suitable culture conditions, has the power of unlimited reproduction by division without conjugation or artificial stimulation."

The race that I worked with in 1901 was a conjugating race which died out in the 742d generation. Woodruff's long line of over 3500 generations is a non-conjugating race and the two races cannot be compared in regard to vitality, since normal conjugation was prevented in the conjugating race, whereas in the non-conjugating race there has been no artificial prevention of a normal process.⁸

In an extensive paper on physiological variations in *Paramaecium*, Calkins and Gregory reiterate the same point of view:

To return to Woodruff's race of *Paramaecium aurelia*, we find this important difference between his material and that which Calkins worked with. The *Paramaecium caudatum* which formed the material for the earlier observations was a conjugating line as shown by conjugation tests made from time to time. In this line, therefore, a normal function—conjugation—was inhibited; metabolism weakened steadily until physiological death carried off all of the race not artificially stimulated. Finally, "germinal death" preceded by the degeneration of the micronucleus and the cortical plasm, carried off the last individual. . . . In Woodruff's material on the other hand, the race is non-conjugating, hence no normal function has been suppressed by keeping the individual apart. So far as known the life history of such non-conjugating forms has never before been undertaken and Woodruff has shown that they will live for at least five and one-half years.⁹

The fact that conjugation has now occurred in mass cultures from my pedigreed race demonstrates that the race is a conjugating race when the proper conditions for its consummation are realized. Therefore there is no evidence extant that a non-conjugating race of *Paramaecium* exists. One who now would demonstrate its existence must plan to carry a race for far more than 4102 generations without a tendency to conjugation being manifested.

⁸ G. N. Calkins, Proc. Soc. for Exper. Biol. and Med., vol. 10, 1913, p. 67.

⁹ Calkins and Gregory, loc. cit.

THE SUPPOSED EXPERIMENTAL PRODUCTION OF HEMOLYMPH NODES AND ACCESSORY SPLEENS

V. STUDIES ON HEMAL NODES

*

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For some decades it has been customary to speak of at least two types of lymph glands or nodes—the ordinary lymphatic gland and the hemolymph node. Although these two types of nodes have been said to occur in man (2, 3, 5, 11, 17, 20, 21, 28, 38), monkeys (17, 37, 37 a), rodents (18, 36, 44), most of the domestic animals (2, 3, 4, 5, 6, 11, 12, 18, 21, 29 a, 36, 37, 38, 39, 44, etc.), birds (18, 36, etc.), fish (36, 37 a), and so forth, those who studied them in greater detail soon found that neither gross nor microscopical differentiation was possible because all manner of transition or intermediate forms were thought to exist. Vincent and Harrison (42), for example, make the following statement:

We believe that we have found gradual transitions from hemolymph glands on the one hand, to ordinary lymphatic glands, and on the other hand, to the structure of the spleen. So that no hard and fast line can be drawn marking off these structures from one another. A lymphatic gland has only to contain blood in part or the whole of its sinuses to constitute itself one of the varieties of hemolymph glands. In certain 'accessory spleens' moreover, the splenic reticulum is so widened out as to approximate to a blood sinus of a hemolymph gland and the Malpighian bodies are so diffuse as to resemble the lymphoid portions of the blood-lymph glands. So that spleen, hemolymph glands and ordinary lymphatic glands form almost a continuous series.

A similar opinion was subsequently expressed by Warthin who further satisfied himself that hemolymph nodes can be produced experimentally and that there is a transition from fat to hemolymph nodes to lymph glands and back again to fat.

In spite of the confessed impossibility of distinguishing between lymph and hemolymph nodes Lewis and Warthin formulated rather elaborate classifications in which the lymphatico-hemal structures of the body were divided into lymphatic, hemal-lymphatic, and hemal nodes. According to Lewis, two types of hemal nodes with an intermediate form, and three types of hemal lymphatic glands can be distinguished. Warthin (46) suggested a further division in spleno- and marrow hemolymph nodes and Morandi and Sisto (25) divide human hemolymph nodes into six groups even. Weidenreich (51) also distinguished several types of hemolymph nodes but upon the basis of injections nevertheless concluded that hemolymph nodes as a class have no connection with the lymphatic system. The latter was, however, denied by Helly (19) and Forgeot (11) who claimed that hemolymph glands did not only contain lymphatics but that all manner of transition forms could be found in which lymphatic vessels simply were near the hilus, in the hilus, and in various stages of penetration of the node. A similar position is also taken by v. Schumacher (4) who concluded that a lymph node may become a hemolymph node and finally perhaps again a lymph node by the disappearance and perhaps the re-entrance of lymphatics. v. Schumacher also considered the hemal node as a rudimentary form of ordinary lymph nodes and divided the latter into those with and without lymphatics.

As will be reported elsewhere, a very large series of injections directly on the carcasses of sheep, has led to the positive conclusion that Weidenreich was correct when he decided as a result of similar injections that the true, not the so-called, hemolymph nodes have no connection with the lymphatic system. If this is true it follows, of course, that this is a simple and crucial test for differentiating lymph and hemolymph nodes although v. Schumacher denies the latter. By means of it the alleged production of hemolymph nodes under various conditions can also be tested easily. If, for example, certain *apparently* newly-formed nodes are found or if lymph nodes have *apparently* been transformed into hemolymph nodes, the identity of the former and the absence of lymphatic connections in the latter can readily

and positively be determined by means of the injection method. It is clear, of course, that no lymph node could become a true hemolymph (hemal) node without being deprived of its intrinsic lymphatic circulation and its connection with the lymphatic system. Furthermore, the vascular circulation of lymphatic nodes would also have to become altered very profoundly before it could even remotely simulate the vascular circulation of hemolymph (hemal) nodes, a fact wholly disregarded by v. Schumacher. Besides certain modifications in the cellular content would also have to occur.

It will be recalled that Tizzoni (35 and 36, et seq.), Eternod (7) and others described splenic nodules on the peritoneum and great omentum which they regarded as having formed as a result of physiological compensation after deficiency in function of the main spleen. Griffini (15) reported similar results and believed new spleens formed from the omentum of the main spleen but Foa (8 and 9) on the contrary regarded these so-called newly formed accessory spleens as normal variations the existence of which Tizzoni had overlooked. However, Warthin (48) referring to Tizzoni's experiments says that the latter's "description of their formation in adipose tissue is confirmed in every detail by the findings in the above cases. (Splenectomies.) His conclusions alone must be regarded as erroneous." Tizzoni's conclusion was that accessory spleens were formed *de novo* while Warthin concluded as Morandi and Sisto (25) had previously done, that the splenic nodules found by Tizzoni, Eternod, Griffini, Mosler and Winogradow were not accessory spleens at all, but hemolymph nodes present before operation but which Warthin considered as newly formed. The cases to which Warthin refers are splenectomies on goats and sheep which resulted in a "new formation of hemolymph nodes from adipose tissue and the transformation of hemolymph into ordinary lymphatic glands." This new formation of hemolymph nodes in fat was observed by Warthin within one month after splenectomy. Subsequently to these experiments Warthin¹ further reported the formation of

¹ Program of Am. Assoc. Path. and Bact.; 11th Annual Meeting. Chicago, 1911.

hemolymph nodes in a dog after splenectomy but I have unfortunately only been able to find the report by title. Vincent (43) upon examining a dog 16 months after splenectomy stated that "There was certainly no increase in the number or size of the ordinary lymphatic glands, but there were some half dozen (rather more than the usual number) of the intermediate form referred to above, i.e., which showed some signs, even to the naked eye, of containing blood." Nevertheless, in order to satisfy himself further regarding this matter Vincent performed splenectomies upon five dogs varying in age from approximately one to two years. From these animals which were killed from five to six months after operation, Vincent reported as follows: "In none of them could I be sure that there was any increase in size and number of the hemal lymphatic glands, nor could I assure myself that of those present any larger portion than usual was occupied by a blood-sinus instead of a lymph sinus." These observations of Vincent seem to confirm the *findings* though not the conclusions of Tizzoni (35 and 36). The latter found reddened lymph nodes in only two out of four splenectomized dogs. In dog III one reddened node was found in the abdominal and thoracic cavities. That in the former had a reddish medulla only but that in the thoracic cavity was reddish brown throughout. All external lymph glands were normal. In dog IV the latter was also the case but the abdominal lymphatic glands which were of normal volume had a very red medulla while the thoracic glands near the termination of the thoracic duct were large and red throughout. Strangely enough from the findings in two out of four dogs Tizzoni (40) concluded as follows:

Les alterations que nous avons constatées dans les glandes lymphatiques internes abdominales et thoraciques consistaient en un grossissement de ces glandes et dans une coloration rouge intense de leur substance médullaire. Ces alterations étaient toujours plus prononcées dans les glandes du côté gauche que sans celles du côté droit, et d'autant plus marquées, que le temps écoulé depuis l'opération était plus court; elles étaient toujours plus accentuées dans les glandes situées près de l'embouchure du canal thoracique. A l'examen microscopique ces lésions étaient représentées par une forte dilation des sinus se la substance médullaire à la suite de l'accumulation de globules rouges libres, de cellules globulifères et pigmentifères, abstraction faite des cellules lym-

phatiques normales; dans les cas les plus anciens on voyait encore des cellules globulifères et pigmentifères entre les colonnes médullaires, et en outre on remarquait une prolifération du tissu conjonctif qui entoure les vaisseaux. Ces glandes lymphatiques présentaient la réaction bleue du Fe. libre plus constamment et d'une façon plus intense que les glandes normales. Nous croyons devoir attribuer la tuméfaction de ces glandes lymphatiques et les autres altérations qui l'accompagnaient à des phénomènes irritatifs simples, à une lympho-adénite consécutive à la lésion traumatique, selon l'opinion de Legros. Les glandes lymphatiques externes et la glande thyroïde n'ont jamais présente de lésions attribuables à la splénotomie.

Foa (8) was the first to question the validity of Tizzoni's (35, 36) conclusions regarding the new formation of accessory spleens after splenectomy. Tizzoni it will be recalled excised the spleen of four dogs and found 60 to 80 accessory spleens in the great and gastro-hepatic omenta in 2, 54 and 90 days respectively after operation. Although no examination of these animals had been made before operation, Tizzoni for wholly insufficient and incorrect reasons, nevertheless concluded that these spleens had formed *de novo*. Foa who had also called attention to the fact that such small nodules as described by Tizzoni are also found in dogs with entirely *normal* spleens apparently did not publish anything further on the subject, but Tizzoni published the results of forty necropsies a few weeks after the formal presentation of Foa's suggestions. In this paper Tizzoni (37) laid special emphasis on the fact that these necropsies were done at the veterinary school of Bologna in the presence and with the aid of his excellent friend Gotti.² The astonishing thing is that although 262 accessory spleens were found in *four* out of the *forty* dogs examined, Tizzoni nevertheless says that all his previous conclusions are confirmed! Tizzoni believed that in these four dogs nature herself had done a partial splenectomy by the development of a chronic interstitial splenitis. Moreover, he performed a splenectomy on a dog that had accessory spleens and although this animal died of an infection before the "usual results could manifest themselves" he nevertheless concluded that the results obtained in this animal also were sufficient wholly to

² The inference is clear, of course, and I mention these details simply because Tizzoni's experiments have been accepted for decades.

warrant his former conclusions! Tizzoni also failed to specify what the results in this case were and then stated that the accessory spleens which form as a result of splenectomy always arise as Malpighian corpuscles which become surrounded by pulp later while on the contrary, in the case of spleens that arise as a result of pathological conditions, the pulp forms first and the rest of the spleen from it.

In May, 1882, Griffini (15) reported a series of experiments on the regeneration of small experimentally-produced defects of the spleen in fourteen dogs and added that in a few cases he noticed a new formation of spleens from the great omentum or from the spleen itself *because of conditions not yet determined*. However, Griffini also failed to give any further details regarding these accessory spleens.

A year later Tizzoni (39) reported that he did splenectomies on two dogs which possessed accessory spleens before operation "as a result of disease." In one animal which was killed six months after operation there was no increase in size and number of the accessory spleens previously present in the great and gastro-splenic omenta but many newly formed spleens were found in the gastro-hepatic omentum, in the lateral ligament of the bladder, in the plica Douglasii, in the ischio-rectal fossa, in the serosa of the stomach and in the central tendon of the diaphragm. In the second animal killed seven months after splenectomy, Tizzoni concluded that there was *perhaps* an increase in size and number of the accessory spleens previously present in the great and gastro-splenic omentum and that in addition other newly formed spleens in all stages of development were found as in the previous animal. Since the first animal had suffered from post-operative local peritonitis Tizzoni concluded that these inflammatory processes inhibited the formation of accessory spleens in the gastro-splenic and great omentum and lead to their formation elsewhere.

Even granting that Tizzoni was still unbiased and not on the defensive it is perfectly evident that all the additional accessory spleens which Tizzoni regarded as having formed *de novo* because of splenectomy may just as well have formed because of condi-

tions present before operation. Hence splenectomy may not have been the causal factor at all. The same objection holds regarding the alleged increase in number of the spleens.

A year later Tizzoni (38) published a series of conclusions regarding accessory spleens drawn from sixty autopsies but he gives no details. These conclusions repeat all the older conceptions regarding accessory spleens and emphasize the fact that the non-experimental new formation of accessory spleens always results from a chronic interstitial splenitis. Tizzoni also drew further distinctions between spleens formed experimentally and as a result of pathological conditions but, since no new facts are presented in this two-page summary and since many of Tizzoni's conclusions can easily be shown to be untenable they will not be discussed here.

During June, 1883, and in 1884 Griffini and Tizzoni (16) reported that *in some instances* they found newly formed nodules on the main spleen and in the great omentum in a series of ninety-seven partial excisions of the spleen. The pieces excised were small (4 to 15 by 5 to 20 mm.) and the dogs were killed from forty hours to eighty-nine days after operation. In this article which the authors call a *résumé* they give no details whatever nor do they rule out reddened lymph glands. This joint *résumé* was followed by another *résumé* by Tizzoni (40) in which he declared that he wanted to test especially what would happen if splenectomy were done on dogs in which accessory spleens had previously formed as a result of disease of the main spleen. These experiments were regarded necessary in spite of the fact that Tizzoni had at least on two previous occasions tested experimentally and reported definite—very definite—conclusions regarding this very matter. Tizzoni again gave no details regarding these dogs—how they were selected, how many and how large the accessory spleens, how these were measured and where located, and so forth, yet he reported that he found an increase in the number and volume of the nodules previously present in the gastro-splenic and great omenta of the two dogs on which splenectomy was done. The experiment was limited to two dogs
“Vir la difficulté d'avoir des chiens qui se trouvent dans les con-

ditions requires." The same objection urged above applies, to be sure, to these two experiments for in these cases also, the conditions responsible for the formation of accessory spleens before operation may have been responsible for their continued formation after splenectomy unless one assumes as Tizzoni apparently did not that these processes are limited entirely to the main spleen for Tizzoni reported the existence of identical pathological processes—chronic interstitial splenitis—in accessory spleens. But it is much more likely that Foa was correct when he confirmed Luschka's conclusion that accessory spleens are also found in the presence of wholly normal spleens.

In a final article Tizzoni reported on twenty-nine dogs in only four of which he found accessory spleens and never more than three in any one animal. The interesting thing in this article is the finding of the same pathological processes in the accessory as in the main spleen and the existence of a zone of infiltration around some of the accessory spleens. Tizzoni again concluded that the findings confirm all his previous conclusions.

Although Mosler (26) is usually quoted as having concluded that a new formation of spleens occurs after splenectomy this is a mistake. Out of thirty splenectomies on dogs done by Mosler the great omentum was covered with dark red nodules in only one dog! These nodules which Mosler upon Roth's examination and diagnosis which was based on Virchow's 'aufgestellten Principien,' called teleangiectatic hemorrhagic lymphomata were noticed ten months after operation. They varied from a lentil to a bean in size and looked like the spleen. Even granting that these nodules were accessory spleens there is absolutely no evidence to show that they were formed after or because of splenectomy. Mosler did not examine these dogs—or any others—before operation and facts presented elsewhere make it highly probable that these nodules were present before operation. Moreover, Mosler himself emphasized the fact that although Roth found hyperplasia of the mesenteric and of the aggregate lymph nodes in this dog he never saw such things in any of the other twenty-nine.

The conclusions of Tizzoni are also contradicted by Vulpus who found no enlargement of the lymph nodes and no accessory spleens after splenectomy in rabbits and goats up to five months after operation. Vulpus also called attention to the fact that Tizzoni failed to produce accessory spleens in rabbits and that Pouchet showed that animals possessing no lymph nodes can survive splenectomy.

From what has been said it is clear that although none of these investigators possessed any reliable criterion for the differentiation of lymph from hemolymph nodes yet the validity of their experiments was absolutely dependent upon the possession of such a criterion. Neither did most of them keep controls. Vincent "In order to institute a fair comparison studied the hemal lymphatic glands in a dozen normal dogs. The average number found was 4 or 5." However, in a footnote Vincent (43) says: "I state this average with some diffidence, as the variations were so great in different cases." But even if the use of controls and a reliable means of differentiation between lymph and hemal nodes could be dispensed with this cannot be said regarding reliable data on the occurrence of accessory spleens in apparently normal dogs. Elsewhere* it has been reported that a large percentage (10+) out of an extensive series of apparently normal dogs and cats possess accessory spleens or true hemal nodes. This being the case it becomes highly probable as Foa first suggested that the splenic nodules found by Tizzoni, Eternod and Griffini on the peritoneum and great omentum after splenectomy were present in these animals before the operation. Morandi and Sisto for other reasons also surmised that the newly-formed nodes reported by these investigators were hemolymph nodes normally present in dogs. Besides Tizzoni's investigations contradict his conclusions. Nevertheless Warthin believed that these and the nodules mentioned by Mosler and Winogradow were hemolymph nodes which were newly-formed from fat and not accessory spleens. It is interesting in this connection to note that Roth who made the pathological

* Meyer, The occurrence of supernumerary spleens in dogs and cats with observations on corpora libera abdominales. Anat. Rec., Philadelphia, 1914.

examination for Mosler especially emphasized the fact that "das sehr sparsam vorhandene Fettgewebe zeigte nirgends eine nähere Beziehung zur Entwicklung derselben." Moreover Winogradow (53) only reported enlargement and reddening of lymph nodes and not a new formation of anything. The above criticisms also apply, of course, to Warthin's conclusions regarding the production of hemolymph nodes by splenectomy for he too did not state that he took the precaution to carefully examine the abdominal cavity of the animals before the operation was done. Moreover, Warthin did not consider the occurrence and presence of accessory spleens in normal animals and emphasized the impossibility of distinguishing between lymph and hemolymph nodes.

But even granting that these considerations do not wholly invalidate the conclusions of investigators who reported positive results the negative results of Vulpus (45), Vincent (43), Piltz (28) and Gibson (14) together with those presently to be reported in addition to other considerations certainly would seem to do so.

Piltz who concluded that there are no hemal nodes in cats and dogs removed the spleen from four dogs but failed to find the formation of hemal nodes in from eight days to three months after operation. Gibson also reported that he found "No naked eye abnormal appearances with the exception of the absence of the spleen" in a dog from which the spleen had been removed five and a half months previously. Similar observations were also made by him on the body of another dog in which both spleen and thyroid were removed. In this animal Gibson stated that he thought that the mesenteric nodes were large as compared with the normal but "not decidedly redder than usual." Practically the same results were obtained by Gibson in another dog from which the spleen had been removed and which had been bled 0.5 and 8 per cent of its body weight respectively within an interval of six days. Regarding this animal Gibson stated that the abdominal lymph nodes were "if anything somewhat redder in the center than usual," and contained some nucleated erythrocytes. However, that the slight—if any—red-

dening thought to be present in the lymph nodes of this dog, was probably not the result of splenectomy is suggested by the fact that in another dog which was bled 2.6 per cent of its body weight three days previously the lymphatic glands were said to have been "perhaps a little larger than usual but were no redder." Nucleated erythrocytes were again seen in scrapings from the mesenteric glands and the author came to the conclusion that erythrocytes are normally found in lymph nodes. .

Meek (24) on the other hand reported that "the experimental production of such (hemolymph) glands in the guinea-pig is easy of accomplishment." Meek obtained these results by the injection of toxic substances such as living pathogenic bacteria, diphtheria toxin, oxalic acid and distilled water. Although Meek defined a "hemolymph gland as one which possesses a sinus containing blood instead of lymph" it is clear that he did not recognize that hemolymph or better hemal glands, have no relation to the lymphatic system whatever for he continued: "It would appear that in pathological conditions in the human subject their occurrence in large numbers is almost universal." Furthermore, farther on Meek stated that since hemolymph glands are only transformed lymph glands and since all manner of transition forms exist between the two there is no justification for a separate category. Hence, it must be clear that what Meek produced so easily in guinea-pigs was not hemolymph nodes at all. Not organs *sui generis* as Drummond first claimed for hemolymph nodes but merely changes present, even if not to the same extent, in normal lymph glands. Such changes no one will deny for as everyone knows it is an old, old observation, indeed, that various cellular changes and the accumulation of blood cells in the sinuses of lymph nodes occur under many pathological and other conditions but that will fail to convert a lymph node into a hemal node. In his experiments on guinea-pigs Meek also failed to state the amount and the strength of the solutions used, the age and weight of the pigs and the interval between the injection and the production of hemolymph nodes.

Since all the evidence for the experimental production of hemolymph nodes and accessory spleens either *de novo* or from

lymph glands, seemed wholly conflicting, a series of experiments on dogs and guinea-pigs was undertaken in connection with studies on hemolymph nodes of domestic animals. Ten dogs taken from two litters with known dates of birth were placed under laboratory conditions as soon as weaned, kept in identical surroundings and fed upon the same food until approximately six months old. The spleen was then removed from eight, one animal from each litter being kept as a control. Before removing the spleen the omenta, mesentery and as far as possible also the whole abdominal cavity, were inspected in order to note the condition of the lymph nodes and the presence of accessory spleens. After operation the animals were again kept under identical conditions and killed 41 to 126 days later, one animal being killed every two weeks. The spleens were inspected for scars and weighed. Recovery in every case was rapid and healing per primam except in one dog in which several small cutaneous stitch-abscesses developed. There were no adhesions in any animal. All of them gained markedly in weight not only because they grew rapidly but because they became fleshy and some even fat. Table 1 gives the age, the post-operative period and the result. The gain in weight is not given because it could have no special significance since all were young and growing dogs.

As is evident from the table, the results on these dogs fully confirm those of Vincent and of many operators on man. Only a single lymph node with a reddened center and 3.5 mm. in size, was found in the gastro-splenic omentum of one dog at the time of operation. No hemal nodes or accessory spleens were seen before or after operation, and it is especially interesting that the above node became paler or at least was temporarily paler, at the time of necropsy fifty-three days after operation. Although it could not be experimentally determined before or at the time of operation, whether the pinkish node was lymphatic or hemal in character this precaution was not neglected at the time of necropsy. All questionable appearing nodes which suggested intermediate forms were injected, but not a single hemal node was found. Since all nodes except those on the head, dorsum

and at the extremities were examined it follows that there should not be any question for skepticism as to the validity of the results in these cases. Moreover, to date over seventy dogs and approximately one hundred cats taken at random, have been examined in order to obtain a basis for reliable judgment

Only a few specimens of the lymph nodes taken from dogs from which the spleen had been removed, were examined micro-

TABLE 1
Splenectomies on dogs

NO. OF DOG	SEX	AGE IN MONTHS AT TIME OF OPERATION	INTERVAL IN DAYS	DEATH BY	RESULT
1	Female	5½	30	gas	No hemal nodes or accessory spleens; bronchial lymph nodes intensely red ¹
2	Female	5½	41	bleeding	No hemal nodes or accessory spleens; lymph nodes normal in appearance
3	Female	5½	53	gas	No hemal nodes or accessory spleens; bronchial lymph nodes intensely red
4	Male	5½	77	gas	No hemal nodes or accessory spleens; all lymph nodes normal in appearance
5	Female	6	89	gas	Same as 2, 4
6	Female	6	98	gas	Same as 2, 4, 5
7	Female	6	112	gas	Same as 2, 4, 5, 6
8	Female	6	126	gas	Same as 2, 4, 5, 6, 7

¹ The necessity for injecting the lymph nodes precluded a satisfactory microscopical study, of course.

scopically. In these nothing unusual was recognized and this was also true as to gross appearances of the lymph nodes in all the animals operated on except two. Moreover, the lymph glands in the two controls and in other dogs of similar ages were in all respects similar in gross appearance. Vincent (43) too concluded that "In spleenless dogs there was no apparent change in the lymphatic glands of any part of the body, neither in the direction of hypertrophy nor increased redness. On microscopical examination the hemal lymphatic glands presented the features of those in the normal dog." In a footnote Vincent adds:

The research has at any rate taught me the enormous difficulty of judging of hypertrophy of a tissue which is distributed in different parts of the body. In the case of the "hemal lymphatic" tissue the difficulty is increased tenfold by the fact that not whole glands but only parts of glands are formed of it, and so counting affords little guidance. It was suggested to me that I should freeze the gland, then cut out and weigh the red portions. This seemed feasible, but nevertheless an attempt was found too difficult and laborious to be practicable.

It will be recalled that many of the older experimenters reported enlargement of the lymph nodes and several also enlargements of the thyroid after splenectomy. According to Vulpian who found no enlargement up to five months in goats and rabbits, Tiedemann, Bardeleben, Gmelin, Mayer, Hyrtl, Domrich, Fisher, Ludwig, Eberhard and Gerlach noticed such an enlargement in dogs and Hegar and Simon in three cats. Zesas reported similar results for three rabbits, Winogradow⁴ in three dogs, and Warthin in goats and sheep. Many of these investigators also reported changes in color and consistency as well as in cellular content and in many instances the enlargement was observed in all the peripheral nodes as well. However, since most of these observers did their experiments in the early days of asepsis Schiff's suggestion that the changes noted in many cases by the early experimenters were due to infection is undoubtedly correct. This suggestion is also supported by the presence of enlargement in *all* the lymph nodes, by the enlargement of the thyroid, by the large percentage of deaths and by the description of the microscopic appearance of the nodes themselves by many of the earlier experimenters. However, Freytag (11) concluded that in the rabbit which possesses no hemolymph nodes, splenectomy is followed by reddening of the lymph nodes near the greater curvature of the stomach and at the ileo-coecal junction in sixteen weeks. Freytag also surmised that this reddening is probably preceded by a swelling of the nodes since the superficial lymph nodes show this reaction and adds that red lymph nodes which are developed from 'preformed Anlagen' can be produced (?) decidedly earlier—within four weeks—by irritation from bleeding.

⁴ Winogradow's percentages are wholly unconvincing for reasons obvious to anyone who examines his article.

Freytag further reported that lymph nodes respond as early as five weeks after splenectomy, that after bleeding in the rabbit small (0.75 cm.) "preformed splenic Anlagen" are found near the hilus of the main spleen, and that if these are removed at the time of operation negative results are obtained. He also stated in contradiction to the above investigators that red lymph nodes develop from the *mesentery* sixteen to nineteen weeks after splenectomy in a place one-half centimeter from the place where the spleen was excised, and that such can be produced in four weeks by bleeding. Still farther on it is stated that glands develop from *fat* and Freytag thinks hemal nodes may be transformed into spleens easier than lymph nodes.

It was stated above that although hemolymph nodes were not formed in any of these dogs in two instances nodes of unusual appearances were nevertheless found. In dog 1, for example, a female killed by illuminating gas, thirty days after operation, the pubo-inguinal, abdominal, axillary and cervical lymph nodes were normal, but two large ($1 \times 0.75 \times 0.5$ cms.) bronchial nodes lying dorsal to the root of the left lung were intensely red and all the visible afferent and efferent vessels engorged with blood. The many—about a dozen—fine, straight, afferent trunks which were only 0.25 to 0.75 mm. in caliber and about 1.5 cm. long were plainly visible up to the lung which they entered. Two larger (1.5 mm.) efferent trunks which were likewise engorged with blood, left these two nodes and joined the larger mediastinal vessels which empty into the main lymphatic vessels near the jugulo-subclavian junction. There also were two reddish nodes similarly placed on the right side. These had similar connections but the efferent trunk of one joined a more centrally placed mediastinal node near the apex of the thorax before joining the larger collecting trunks. The other node which lay nearer the root of the lung was large (1×0.5 cm.), flat, oval and joined by twelve to fifteen extremely fine blood-filled lymphatics which also seemed to have a sub-pleural origin.

In the other dog—No. III, also a female—killed fifty-three days after operation the pubo-inguinal, abdominal, axillary and cervical nodes were normal in appearance but the bronchial nodes

which were all as intensely red as the specimens in dog I, also had similar connections. Here then would seem to be two instances in which hemolymph nodes were produced experimentally. Not in the abdominal cavity, however, but in the thorax instead. But since very reddish nodes found in apparently normal animals, had always been found in connection with the lymphatic system the injection method was resorted to in these two cases also. By means of puncture of the subpleural tissues on the left side some of the fine straight engorged vessels joining the hemorrhagic nodes in dog I were injected. The india ink could plainly be seen displacing the blood in the fine afferent vessels and entering the node. Some of the vessels that were not injected in this way were however injected in a retrograde manner by puncture of the node which they joined. By this means the engorged efferent vessels were also injected and the india ink followed to its entrance into the jugular vein. The node on the right side was treated similarly with identical results and the same results were obtained in dog III. All the hemorrhagic nodes and vessels were thus shown to be in direct communication with the large collecting mediastinal lymphatic trunks. It is possible, to be sure, that the peculiar condition of the bronchial lymph nodes was due to splenectomy as some of the earlier investigators believed. Although improbable this is not denied, but that does not prove that they were true hemolymph (hemal) nodes which had been formed from lymph nodes or that they were newly formed nodes. As stated above, congested and hemorrhagic bronchial and mediastinal lymph nodes are very commonly found in both dogs and cats. Besides no such nodes were present in dog II which occupies an intermediate position in time, having been killed forty-one days after operation.

Haberer (18) in his study on supernumerary spleens also directs attention to the fact that many of the structures reported as accessory spleens probably are not such. He stated that he confused lymph nodes changed by sepsis, with spleens *macroscopically* although they were found to be typical lymph glands *microscopically*. Furthermore, Haberer says that in emaciated individuals he often found specimens which did not look like

spleens microscopically because they contained no Malpighian corpuscles, nor like lymph nodes but like transition forms resulting from pathological conditions.

Since the primary object of these experiments was not to determine what organs or tissues, if any, act vicariously for the spleen but to test the alleged formation of accessory spleens and hemolymph nodes after splenectomy, no special attention could be paid to microscopical changes which may occur in lymph nodes after splenectomy. The necessity for injecting them precluded such an examination but it may be recalled that Vincent found no changes in the lymph nodes after splenectomy. However, since his examinations were made on dogs five to six months after operation early changes as reported by Freytag may nevertheless have been present. Moreover, since Warthin reported the formation of hemolymph nodes in the abdominal cavity as early as a month after splenectomy, this observation and the above findings may undoubtedly partly be accounted for by such early changes in lymph nodes. This supposition also receives some support from observations after splenectomy by Winogradow (53), Zesas, Hegar and Simon and others.

In considering the factors responsible for the reddening of lymph nodes and the presence of blood in the sinuses Vincent (44) said: "I have not so far studied the blood-supply to these glands by means of injection, nor have I succeeded in finding the bloodvessels in direct communication with the 'lymph sinuses of the gland, but from the large number of red corpuscles sometimes found in them, I am convinced that there must be such communication.'"⁴ It would be foolish to deny the possibility of the exceptional existence of such intra-nodal communications but from a large series of injections made into such nodes one is prompted to recall that Hyrtl tells us that someone was convinced that the seat of the soul was in the pineal gland but reflectively adds, "He found it not."

Winogradow, who found that the lymph nodes were enlarged soft, juicy, and dark and light red—especially red in the cortex—

⁴ Lewis (21) stated that his observations regarding the existence of such communications are confirmed by himself, by Winogradow and also by Warthin.

so as to remind one of the spleen, because of the accumulation of erythrocytes which lay free in the reticulum among the lymphocytes, believed that this was the result of diapedesis after splenectomy. Tizzoni, however, as quoted above, regarded the changes in lymph nodes due to a lympho-adenitis resulting from a simple traumatic irritation.

In view of Meek's observations and conclusions a series of experiments were also undertaken on five guinea-pigs of known weight. One was given repeated injections of distilled water, the other of oxalic acid and three of diphtheria toxin. The injections were made directly into the peritoneal cavity. The amounts injected and the strengths as well as all other necessary data are given in table 2.

From the table it will be seen that hemal nodes were not found in a single instance. In case of no. 5 which died one week after the injection of 5/7 T the peritoneal cavity contained 1 to 2 cc. of bloody exudate but no hemal nodes had formed. The lymph nodes were difficult to recognize and only a single large lymph node at the root of the mesentery was slightly reddened. On injecting this node the usual results were obtained. The same statement holds for guinea-pig 4 which succumbed one day after the third injection of 1 cc. of a 1 per cent oxalic acid solution, and also for all the other animals.

Since as Retterer (29) showed, it is not uncommon to find more or less reddish lymph nodes in entirely normal guinea-pigs of all ages, under various conditions, and from many injections made on such, I am compelled to conclude that the above experiments did not result in the production of hemal nodes nor did they produce any unusual color changes in the lymph nodes. Hence from a review of the literature and from the experimental evidence available the conclusion that up to the present, no one has offered sufficiently conclusive evidence for the experimental production of true hemal nodes—not hemorrhagic lymph nodes—and accessory spleens is unavoidable. However, since true hemal nodes would *seem* to appear and disappear for some unknown reason the *possibility* of their experimental production—and that of supernumerary spleens?—is not denied. Changes in lymph

TABLE 2

NO. OF ANIMAL	SEX	WEIGHT JUNE 8	INJECTION JUNE 8	WEIGHT JUNE 15	INJECTION	WEIGHT JUNE 20	WEIGHT JUNE 27	WEIGHT JULY 10	INJECTION JULY 10	WEIGHT JULY 18	INJECTION JULY 18	REMARKS	RESULTS
1	M	200	0	235	0	270	295	325	0 5 cc. nor. salt cont. 5/14 T.	372	1 cc. nor. salt cont. 5/7 T.	Well Aug. 10; killed	Results as 4
2	F	230	0	260	0	285	290	230	0	297	1 cc. nor. salt cont. 5/7 T.	Sick July 20; died July 22	Results as 4
3	M	250	2 cc. aq. dest.	280	2.5 cc. aq. dest.	295	295	350	1.5 cc. aq. dest.	428	3 cc. aq. dest.	Well Aug. 10; killed	Results as 4
4	F	250	1 cc. 1/10 per cent oxalic acid	280	1 cc. 1/15 per cent oxalic acid	320	345	390	1 cc. 1 per cent ox- alic acid	Died July 11; no hemal nodes or ac- cessory spleens; lymph nodes apparently normal			
5	M	270	1 cc. nor. salt cont. 5/7 T.	295	1 cc. nor. salt cont. 5/7 T.	250	Died June 20; lost normal to gross appearance; injected					lymph nodes present; lymph nodes present; lymph nodes	

nodes, following various influences, have, of course, long been recognized but the nodes remain what they are—lymph nodes—and are not thereby converted into something else—hemal nodes having no connection with the lymphatic system.

It can be urged against these experiments on guinea-pigs that the injections were not continued over a sufficiently long period and that hemal nodes may have failed to form for this reason. Fortunately through the courtesy of my colleague, Professor Zinsser, I was able to examine the carcass of a goat which had been immunized against typhoid nine months before death and which had also been bled repeatedly. Yet only several small hemal nodes were found. Since all other goats examined during the past years contained more hemal nodes than this animal which had received repeated injections of toxin-containing material and had also suffered repeated losses of blood, it is evident that neither of these things resulted in the production of hemal nodes. Through similar courtesy I was also enabled to examine the carcasses of two sheep, one of which, although only about three years old, had been bled from 5 to 125 cc. approximately a hundred times during a period of two years. The other had been bled forty times during a year. In spite of this repeated loss of blood these animals were very fat indeed. Yet upon examination all lymph nodes were found to be exceptionally pale and small and only about a score of small apparently hemal nodes⁶ from 1.5 to 2.5 mm. in size were found in the fat about the superior mesenteric vein in the first sheep and six in the second. In the first sheep, none were present in the very large amount of fat in the lumbar prevertebral region where they are usually so numerous, and only five (2–3 mm.) in the second. From these things it is evident, of course, that the injection of toxin or the loss of blood, even if repeated and extending over long periods, do not cause the formation of hemal nodes or necessarily produce any marked permanent macroscopic changes in lymph nodes. That these things may evoke a transient reaction on part of the lymph nodes is probable, to be sure, but that does not justify

⁶ These were too small for puncture injections.

the conclusions drawn by many observers regarding the formation of hemal nodes. That changes may be produced in lymph nodes by repeated bleeding is also suggested by the studies of Smith indicating a lowering of the resistance of erythrocytes after the withdrawal of blood, but it is more than likely that the amount and frequency of that loss are very important factors in the determination of the character and extent of the changes in lymph nodes, a conclusion which also seems to be amply justified by Freytag's observations.

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THE OLFACTORY SENSE OF THE HONEY BEE¹

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¹ The second part of this paper, which is a complete discussion of the literature pertaining to the sense of smell in insects, must be omitted on account of its length.

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INTRODUCTION

Most students of bee behavior have considered this the fundamental factor in bee culture and have neglected the mechanisms through which behavior is manifest. Behavior consists only of the reactions or responses brought about through the senses and by means of internal stimuli. It is well to emphasize the necessity of studying the structure and function of sense organs and to form definite opinions concerning behavior only after the morphology of these organs is thoroughly understood. Behavior and the structure of the sense organs are inseparable and should be studied at the same time.

In the investigation here recorded two objects have been kept in view: (1) To determine the relative sensitiveness of the honey bee to different odors, so that it may be expressed numerically for comparison under different conditions; and (2) to locate the olfactory organs. The study of the behavior of normal bees under experimental conditions is used as a basis for correctly interpreting the observations on bees made abnormal for the purpose of obtaining data which concern the two objects in view. Since smell is probably the most important sense inside the hive and is perhaps only of secondary importance outside, we should know all that is possible concerning it.

Some writers assert that bees have an acute sense of smell, while others regard this sense in the bee as not important. Every bee keeper believes that this sense plays an important part in

the lives of his bees, but as yet he is able to profit by such a belief in only a few ways. For example, by the use of smoke he is able to control these insects while working with them. Sometimes a bee keeper uses a strong-smelling chemical while uniting colonies or in other manipulations.

Many entomologists have had something to say about the seat of the organs of smell in insects, but most of the views are purely speculative. A few have done extensive and thorough experimental work to determine the location of this sense. However, since they have failed to study sufficiently the behavior of the insects investigated, the responses observed have misled them in determining the seat of the olfactory organs. It is now generally believed that the antennae bear the organs of smell, but as all the antennal organs are covered with a hard membrane the objection has been raised that such organs can not receive olfactory stimuli. Hicks ('57) discovered some peculiar organs on the bases of the wings and on the legs of insects and suggested that these organs have an olfactory function. They have been neglected since 1860, when Hicks presented his third and last paper on them. About the same time several investigators began to work seriously on the antennal sense organs. This branch of work was later carried on by others, but the olfactory organs of Hicks have seemingly been forgotten.

MATERIAL AND METHODS

The Italian honey bee, mixed more or less with the black or German race, has been used in all the following experiments and observations. For the purposes of this paper workers are divided into four groups according to age; (1) One-day-old bees. They are gray, relatively inactive, and do none of the work of the hive but eat freely; (2) Young bees, between one and four days old. They are active, have begun to do certain work in the hive, and are still rather gray; (3) Middle-aged bees, from the age of four days to that at which they become black and shiny. This period includes the greater part of the bee's life. They have lost their gray appearance and the thorax is still quite hairy, having the same general color as other parts of the body; (4) Old bees

having the top of the thorax black and shiny, due to the loss of hair. Many times their wings are ragged at the tips and posterior edges.

To obtain material for the study of the disposition of the organs described by Hicks ('57-'60), workers and drones just emerged from the cells were used. Since queens of this age were not available when needed, older ones were employed. The legs and wings were pulled off at their articulations, and the thoraces and abdomens were slit open. These parts were put into a cold saturated solution of caustic potash, where they remained from one to three days, depending on the size and coloration of the material. When removed from this solution, the material was washed thoroughly in water and mounted between two cover glasses in a one-fifth saturated solution of potassium acetate. This solution gives a good refractive index and at the same time unites readily with the solution of asphaltum used for holding the two cover glasses together. It was thus possible to study the two sides of the specimens under the highest magnification.

To obtain material for the study of the internal anatomy of the organs herein discussed, worker pupae 13 to 21 days old (counting from the time the eggs were laid) were killed. The various appendages were removed and were then cut into small pieces, which were immediately dropped into fixing fluids. Various fixatives were used but the modified form of Carnoy's proved best. This fluid contains equal parts of absolute alcohol, chloroform, and glacial acetic acid, with the corrosive sublimate to excess. The material was left in this solution $5\frac{1}{2}$ hours. It was dehydrated in the ordinary way but was cleared with cedar oil by using the sinking method. In order not to harden the chitin too much the material was left not more than two minutes each in 95 per cent and in absolute alcohol and not more than 10 minutes in cedar oil. It was then placed for 10 minutes each in ether and in thin celloidin, after which it was put into a vial containing thicker celloidin. The vial was put into melted paraffin and was watched closely, for the celloidin solution boils at a low temperature. It was boiled slowly for 10 minutes, until the celloidin became thick. The material was then removed from the vial, and a small mass

of the celloidin surrounding each piece was allowed to remain in the open air until the celloidin became firm. The pieces of material embedded in celloidin were then placed in a second vial containing a solution of three parts of chloroform and one part of 55° M. P. paraffin. It was again boiled for 10 minutes in the same manner as described above. It was then removed from this solution and infiltrated for 10 minutes in 55° M. P. paraffin inside the paraffin bath and finally was embedded in the same paraffin.

Serial sections were cut 6 and 10 microns thick and were mounted on slides in the usual way. These sections were stained on the slides with iron hematoxylin and eosin and also with safranin and gentian violet. The former double stain is the better for sense cell differentiation, but had it not been for the use of the latter double stain, the manner in which the sense fibers terminate would not have been clear.

When all the foregoing steps were carefully followed, good serial sections were had in abundance, but none were perfect, owing to the hard chitin. In fact these were only celloidin sections embedded in paraffin, although other celloidin sections, 35 microns thick, of entire adult workers were made by following the usual celloidin method. These were stained with Ehrlich's hematoxylin.

The drawings were made at the base of the microscope with the aid of a camera lucida, and all are original except figure 24, which was copied from Schenk ('03) to illustrate the anatomy of the antennal organs.

EXPERIMENTS ON BEES IN AN OBSERVATION HIVE

In order to ascertain the sensitiveness of workers to various kinds of odors in their natural environments, for comparison with the results in observation cases, an observation hive was constructed from a 10-frame Langstroth hive. The ends and sides were replaced by glass except a mere framework, the two end glasses being fastened permanently, while on each side were two pieces of glass so arranged as to slide. Thus an opening of any size could be made on the sides so as to expose the bees on the combs. The ends and sides had wooden shutters which fitted snugly against the glass, making the interior of the hive dark,

as in an ordinary hive, except when the shutters were removed so that the bees could be observed. This observation hive was mounted on a bench 3 feet high.

A little piece of cotton wet with oil of peppermint was placed on the alighting board among the workers. All of them except the fanners scattered in only a few seconds. The fanners, however, very seldom moved, but continued to vibrate their wings rapidly. Perhaps the current of air which each fanner created prevented the odor from coming in contact with its olfactory organs.

When this piece of cotton was held close in front of the bees, they always vibrated and stroked their antennae with the first pair of legs, turned around quickly, and moved away hurriedly. Also, whenever an open vial of peppermint was held near them, they always fled in a few seconds.

Three small pieces of cotton, one wet with oil of wintergreen, another with water, and the third with sugar sirup, were placed on the alighting board among many workers. The bees did not come closer than within $1\frac{1}{2}$ inches of the first, they surrounded the second and sucked the water, while they literally covered the third piece of cotton to get at the sirup. When the cotton wet with sirup was placed against the one moistened with wintergreen, the bees sucked at the sirup only on the side farthest from the oil.

On three consecutive days in July three lots of eight dead workers each were placed on the alighting board to ascertain how long it would take the workers to remove them. The time varied from 35 seconds to 7 minutes and 45 seconds with an average of 3 minutes and 11 seconds for all 24 bees. A small drop of oil of peppermint was then placed on the tops of the thoraces of these same dead bees. Eight at a time were put on the alighting board as before. As a rule the workers never came nearer than $1\frac{1}{2}$ inches of the scented bees and when they came this close they usually vibrated their antennae, turned to one side and went away. Occasionally a worker would rush up to one of the dead bees, seize it by a leg, wing, or antenna, loosen its hold as soon as possible, and jump backward. As usual, when a worker

seized one of these dead bees it had trouble in freeing itself because of the interlacing of their hairs. The mouthparts of the worker never came in contact with the oil of peppermint. After some time every scented bee was removed from the alighting board. The time taken to remove them varied from 10 seconds to 36 minutes, with an average of 10 minutes and 19 seconds for all 24 scented bees. Because of the repellent odor the bees were three times as long in removing the bees so treated as in the case of the untreated bees.

A small piece of cotton wet with oil of peppermint was held $\frac{1}{2}$ inch from the bees that thickly covered one of the combs in the observation hive. The time which elapsed before the bees had scattered, leaving a circular space with a diameter of 3 inches, was recorded. This experiment was performed 20 times with intervals of 20 minutes between each two tests. The time varied from 10 to 20 seconds, with an average of 16 seconds. Similar experiments were performed with chemically pure oils of thyme, clove, wintergreen, and cedar. The results obtained are as follows: Oil of thyme 10 to 25 seconds, average 16 seconds; oil of clove 15 to 45 seconds, average 21 seconds; oil of wintergreen 15 to 30 seconds, average 22.5 seconds; oil of cedar 20 to 45 seconds, average 29.5 seconds. The general average for the 100 experiments with all five oils is 21 seconds. Judging from these results, peppermint and thyme have an equally repellent odor to bees. To bees, as well as to human beings, cedar oil has the least repellent odor of all five of these oils.

EXPERIMENTS ON NORMAL BEES IN OBSERVATION CASES

To determine the relative sensitiveness of queens, drones, and workers to various odors, under conditions which permitted of their close observation, triangular cases were used. These were made of three narrow wooden strips, two of which were 10 and the third 6 inches long, each strip being $\frac{1}{2}$ inch thick. Cheese-cloth served as a bottom and glass as a top for the case. The apices and bases of these cases rested on two supports above a rigid table and the table legs rested on a concrete floor, near a

window. No screen was used to prevent the bees from seeing the observer because they never showed any response to movements other than rapid ones.

Nine middle-aged workers marked by clipping the wings in various ways were placed in each case. A lump of queen-cage candy (made by kneading powdered sugar in a small amount of honey) was kept continually in each case. At various times these bees were given water, sirup, or honey on a piece of cotton. To make conditions in these cases more natural for the bees, a small piece of comb was inserted.

Queens were introduced at various times and now and then one or more drones. Queens were tested while they were in observation cases with workers, but drones were tested when several of them were in the same case without workers.

WORKERS

To learn whether the results obtained in experimenting with bees in observation cases may be considered as normal, it was necessary to study the behavior of bees in these cases and to compare this behavior with that of bees in an observation hive. The behavior of individual bees in such captivity is in many respects similar to that of bees in the hive. With only nine in each case, it is possible to study the peculiarities in the behavior of each individual bee and each detail may be recorded, along with the bee's number. In these observation cases middle-aged workers live from 3 to 24 days, with an average of 9 days and 3 hours; queens 12 to 27 days, with an average of $16\frac{1}{2}$ days; drones 6 hours to 13 days, with an average of 3 days and 9 hours.

When introduced into the observation cases workers are extremely restless and go about touching everything with their antennae. In a few minutes they usually begin to eat the candy, and after that they behave more normally. They are always more or less restless, and half of them are never perfectly quiet for even a few seconds, except when eating. Some bees eat during nearly the entire day, while others eat little at any time. The majority eat more frequently in the forenoon and are more restless in the afternoon.

They quite often clean and feed each other and stroke with their antennae the antennae and other parts of the body of the other bees in the case. They frequently crawl over the piece of comb and into the cells. Fanners and guards are often noticed; the former sometimes create air currents that can be easily detected, and the latter always stand at the edge of the group of bees ready to jump at any intruder.

When the nine bees introduced are from the same hive, no fighting is ever observed among them, unless one acts "crazy" by whirling around and around, in which event the others are apt to attack it. When a second lot of bees from the same colony, taken three or four days later, is introduced among an old lot, fighting is quite common. In case the second lot is introduced in less than three days there is little or no fighting. In all instances in which strange bees are introduced fighting occurs; the strange bee invariably tries to escape, but it never fights until firmly seized, when it fights by biting and trying to sting. In the majority of cases the stranger is stung first, although at times the combatants seem to sting each other at the same instant. In most cases the stranger loses its life, while the attacking bee escapes unhurt, except for bites on the legs and wings.

Forty fanners were removed from the alighting board of a hive and were put into an observation case somewhat larger than those described on page 271, into which candy, a piece of comb, and a piece of cotton soaked in water had been placed. Forty fanners from another hive were introduced into a similar case. Fanners are readily distinguished because they stand facing the entrance and fan with their wings. Nearly every one of the 80 was a middle-aged worker. When introduced into the cases most of these bees busied themselves in eating candy, a few began to fan, a few acted as guards, and the remainder wandered about aimlessly. On the next day several were seen eating, a few fanned, some acted as guards, but the majority ran about. Some of these carried bits of cotton and comb, having become cleaners.

The experiment was repeated with the same number of guards. These workers are also easily distinguished, for they guard the

entrance and jump at and usually seize any intruder. Half of them were old bees. When introduced into the cases most of them ate candy greedily, and the others ran about aimlessly. A day later most of them were quiet, some were seen eating, a few acted as guards, several carried bits of comb and cotton, a few pulled and tugged on the cotton, and the rest wandered about trying to get out. They never fanned except when excited. When either fanners or guards are removed from their hives and are put into observation cases, they cease to perform their special duties but carry on all the various vocations as far as they can under the conditions. This would indicate that in the hive a worker performing a special duty is capable of doing all kinds of work in case of need.

When one-day-old bees are introduced into observation cases they wander about considerably, although quite slowly. After a short time they become quiet and are inactive. They soon learn to eat candy and from this time on become more lively. They live from 17 hours to 13 days, with 5 days and 17 hours as an average.

When both young and old bees are put into an observation case, the young ones generally begin at once to clean the old ones. A young bee meets the old worker face to face, they stroke one another's antennae, and immediately the younger one begins to clean the older one's wings, legs, abdomen, and other parts. Ordinarily young workers clean bees older than themselves.

Very young bees, whether or not they are sisters of the bees already in the cases, are never attacked, and in fact the old bees "pay little attention" to the young ones.

QUEENS

When the nine bees in the observation case have a queen they appear to be slightly less restless and devote much of their time to her. They feed and clean her and usually at least a few are always around her. When a strange queen is put among them, they seem at first to be slightly afraid of her and keep their

distance. In a moment one or two begin to bite her legs and pull her wings, but this rough treatment never lasts long and no strange queen was ever seriously injured in these cases. After a short time the workers treat her as well as they would their own mother.

On many occasions the queens are seen eating candy, and they repeatedly examine the piece of comb. Very often the end of the abdomen is inserted into the cells of the comb, but no eggs were ever found. In most cases when a queen dies the workers no longer "pay any attention" to her, but in one instance after the queen had died she was surrounded for some time by the workers until one of them seized her wing and tried to drag her away.

DRONES

In every instance when one or two drones were put into one of the observation cases the workers attacked them. The drones run about inside the case aimlessly and continually try to get out. They "pay attention" to nothing and often run against one another and against the workers. A worker sometimes follows a drone, biting at his legs and wings. Very often a drone drags one or more workers that are holding tenaciously to one of his appendages around in the case several times. As soon as the workers can halt his wanderings, one of them climbs upon his back and tries to sting him. Perhaps more drones are actually worried to death by the workers continually following and nibbling at their wings and legs than die from the effects of the sting. On one occasion a worker was observed trying to sting a dead drone. A drone never offers to defend himself in any way but endeavors to escape.

When six to nine drones are introduced all at once among as many workers, the latter appeared to be overwhelmed by their number and seldom offer an attack unless one drone becomes separated from the others. Even in such cases a great deal of attacking is witnessed throughout a day, although no one drone is continually attacked until it dies. When nine drones are

placed into a case without any workers they live in peace among themselves.

Those which are continually tormented by the workers usually live only a few hours, while those that are not molested generally live several days. One lived 13 days and 2 hours, while the average for all was 3 days and 9 hours.

When only one or two drones are in a case they are never seen eating, but when several are in each case, and if they are not tormented by the workers, it is quite common to see them eating candy. Out of 99 drones observed only once was a worker seen feeding a drone and in only two cases did one drone feed another.

Of all three castes of bees in observation cases, the workers are the most active. Queens come second but their actions are extremely sluggish compared with those of the workers. Drones are the least active, although when irritated they move about more actively than queens. Drones usually remain more or less torpid, which causes them to appear sluggish.

EFFECTS OF CLIPPING WINGS

Some additional cases of the same dimensions as the observation cases were made with wire-screen bottoms and dark red glass tops and provided with candy. Half of the right wing in 12 middle-aged workers was clipped off and the bees were put into one of these cases. Twelve bees from the same colony and of the same age which did not have their wings clipped were put into a second case with a lump of candy. These cases were kept in a dark table drawer and the death of each bee was recorded. This was repeated several times, and it was ascertained that the bees with clipped wings lived from 1 day and 19 hours to 12 days and 3 hours, with an average of 9 days and 4 hours; those not clipped, from 19 hours to 13 days and 19 hours, with an average of 9 days and 3 hours. Of the former 71 bees were used and of the latter 66.

In each set of 9 bees kept in the first type of observation case, various portions of one or both wings were removed from 8 of

the bees while those of the ninth bee were unmutilated. With all of these clipping showed no effect; this is true for both workers and drones. Of the former 405 were employed and of the latter 99.

To test this further and to ascertain if a greater number of bees in the same case makes any perceptible difference, 100 middle-aged workers were distributed equally in four large observation cases. Both wings of each bee were cut off close to the thorax. The bees always ate freely, seemed normal in every way, and lived from 18 hours to 27 days with an average of 10 days and 15 hours. Judging from these results, clipping the wings of workers and drones does not shorten their lives, and the increase in the number of bees in each observation case makes little if any perceptible difference. We may infer that the clipping of a queen's wings to keep her from going away with the swarm does not shorten her life, as some bee keepers have claimed.

Later many wings of all three classes were studied microscopically and it was found that the base of the wing contains a large nerve, many sense cells, and a tracheal branch, but no muscle extends beyond the base of the wing. When a wing is cut off close to the body, leaving only a short stump, the removal does not injure the structures enumerated above because the scissors pass beyond all of them. This subject will be discussed further in the section devoted to the distribution and structure of the olfactory pores, pages 299 to 333.

EFFECTS OF LIGHT ON LONGEVITY

In the experiments with the bees in observation cases and with those kept in the cases used for the experiments with bees having the wings clipped, no appreciable difference in the length of life is noticed. The bees in the observation cases were always kept in a bright light, while those in the other cases were always kept in a dark table drawer. The average length of life for the bees exposed to light was 9 days and 2 hours and for those kept in darkness 9 days and 3 hours. It may be inferred, therefore, that in captivity light does not shorten a bee's life.

LONGEVITY OF ISOLATED BEES

The statement is often made that when a bee is isolated and can not communicate with other bees it dies within a short time. To test this, 40 middle-aged workers were carefully selected and were placed singly in small triangular observation cases, two of whose sides were 5 inches, and the third side 4 inches in length. The depth was $\frac{1}{2}$ inch; the top was glass, and the bottom was wire screen. These bees always had plenty of candy and were given water occasionally, and the cases were placed side by side on a box by a window. In these circumstances the bees could not communicate with each other by sight or touch, but smell, and hearing—if they hear—were not excluded. These workers seemed normal in every respect and they lived from 4 days and 8 hours to 33 days and 22 hours with an average of 11 days and 20 hours.

The same experiment was performed with 100 active young bees that were just emerging from their cells and had never seen or associated with other bees. One might expect bees which had just emerged to live longer than middle-aged ones. However, this was not the case, for they lived from 1 day and 16 hours to 26 days and 4 hours, with an average of 4 days and 10 hours. The isolated middle-aged bees lived more than twice as long as the young workers.

LONGEVITY OF BEES WITH STINGS EXTRACTED

The opinion is sometimes expressed that when a bee loses its sting it dies within a short time. To learn whether this is true the stings of 100 middle-aged bees were removed by holding a bee by a wing against the fleshy side of a meat rind. The bee stings the meat rind and loses its sting. In every instance the sting with all its accessory parts, including the poison glands, was pulled out. Several times half, and a few times the whole intestine was pulled out with the sting. These bees were introduced in equal numbers into two large observation cases, and were fed candy and given water as usual. Most of them behaved

- normally in all respects by eating freely and by feeding and cleaning one another. Some of them fanned and sometimes one licked the protruding intestine of another bee. Some were very weak and showed signs of dying at once. The length of life after losing the stings varied from 1 hour to 6 days, with 30
- hours as an average. Possibly in the hives a bee with its sting removed may live somewhat longer and may continue its work in a more or less normal way.

EFFECTS OF WEATHER

Climatic conditions perceptibly affect the activity of bees. When it is extremely warm, they are most active and are rarely quiet even for a few seconds. When it is moderately warm, they are less restless, and when rather cool, bees do not move freely. From 8 to 11 o'clock in the morning is the best time for studying the responses of bees to various odors, because they are then less active than during the middle of the day. From 4 o'clock until sundown is the second best period for studying their responses. During cool weather their movements are quite sluggish and when the humidity is high they are much less active and respond to various odors more slowly than when there is a low humidity. During damp weather they seem to live longer in observation cases than when the humidity is low. This may be because increased activity shortens its life.

CAUSES OF MORTALITY IN OBSERVATION CASES

In the observation cases bees apparently die from four causes, as explained below:

1. Old age is the most common cause of death. The greatest precautions were taken in selecting what were considered to be middle-aged bees, but even then many died in these cases within only a few days although they ate well and were no more active than usual.

2. Doubtless many of these bees live a shorter time on account of their extreme restlessness. Some of them were never quiet and did not even take time to eat, while others ate occasionally.

This extreme restlessness without sufficient food was certainly the cause of their shortened life.

3. Fighting is very fatal. It was usually prevented by using bees taken from the same hive at the same time. Often bees which had been separated while fighting never attacked any other bee again, but lived thereafter in peace.

4. Several times, during damp days especially, when sugar had accumulated on the floor of the case, all the bees in a case died within a short time. An examination showed that they had become coated with this wet sugar and were unable to clean themselves. This coating caused them to appear dark several hours before they died and evidently was the cause of their death.

OLFACTORY EXPERIMENTS

The following sources of odor were used for determining the reactions of the bees in observation cases: Chemically pure essential oils of peppermint, thyme, and wintergreen; bee foods, honey, and comb, and pollen from old combs; parts of plants, flowers of honeysuckle (*Lonicera* sp.), leaves and bits of the stem of pennyroyal (*Hedoma pulegioides*?), of spearmint (*Mentha spicata*), and of scarlet sage (*Salvia* sp.); and bee stings. These substances were kept in stoppered vials of the same shape and size. The parts of plants were used only so long as they remained fresh. Bee stings were obtained by seizing workers by the legs with a pair of forceps and holding them to a meat ring. In a few seconds the bee invariably stung the ring and its sting was pulled out. The stings with the adhering poison sacs and muscles were immediately put into a tightly stoppered vial. One hundred such stings were used at a time and give characteristic irritating and pungent odor. At first this odor is similar to that emitted when a bee stings an object, but after a few hours it becomes somewhat different, not quite so irritating but more pungent. In 24 hours this odor becomes foul and unpleasant and after 48 hours it is one of the foulest and most offensive that one can experience. These stings were used not longer than 20 hours after removal.

To ascertain the true responses of bees to the odors of the preceding substances, the chosen insects were carefully observed each day as long as they lived. A bee was tested only when it became perfectly quiet, without even the antennae being moved in the least, and when it was not watching another bee or any other object, and was not eating. The stopper was quickly removed and the vial was then gently and slowly placed under the observation case directly beneath and within $\frac{1}{2}$ inch of the individual being tested. Occasionally the vial was placed a few inches in front of the specimen, to test its ability of smelling for a short distance. Whenever all of these precautions are taken, a normal bee responds to any one of these odors without failure. As a control, an empty and odorless vial was now and then placed under the bees in the same manner. If by chance a bee moves while the control test is being made, its behavior is quite different from that observed when odors are used. Only the first responses have been recorded, and in all cases where there was the least doubt as to whether the bee moved for any reason other than the olfactory stimulus, such movements were never recorded. The reaction time was counted in seconds. With an ordinary watch the minimum time which can be definitely recorded is 2 seconds, although many individuals respond to some of the odors much more promptly. Owing to this source of error the average recorded time is probably double what it should be in the cases where the response was prompt. An intermission of 15 minutes elapsed between any two tests in the same observation case and generally several times this period elapsed before the same bee was again tested with the same or with a different odor. Some individuals were tested five times with the same odor, although most of them were not so tested more than twice. In all, 509 specimens have been carefully observed, but on account of the extreme restlessness of some of the bees it has been possible to obtain records for only 263.

In the following paragraphs are given the responses of the three castes of bees to the odors of the ten different substances and the average reaction times in seconds. In recording the responses the term "vibrate" is used to describe the rapid move-

ment of the antennae up and down or from side to side. When this movement is slow they are described simply as having "moved." When the expression "acts as if noticing odor" is used it is to be interpreted as meaning that the antennae are "vibrated" and that the bee seems to "fix its attention" on the spot from which the odor comes. Anthropomorphic terms are of course used only in a figurative sense, often for lack of words adequately to describe the actions otherwise. Sometimes a bee lies flat on its thorax and abdomen, so the word "arose" is to be interpreted as meaning that the bee gets up and stands on its feet. In the averages of reaction times the probable error is presumably high. It has not been calculated, since slight differences in reaction time are not considered as significant in the discussion of results.

Workers

Oil of peppermint:

- 13 moved away quickly
- 6 moved away quickly and vibrated antennae
- 6 moved away slowly and stroked antennae with front legs
- 4 moved antennae slightly and moved away slowly
- 2 jumped quickly as if attacking an enemy
- 2 turned around quickly and moved antennae
- 1 put its head on floor of case and vibrated antennae
- 1 arose quickly and stroked antennae
- 1 arose slowly and stroked antennae
- 1 arose quickly

Reaction time 2 to 3 seconds, average 2.3 seconds

Workers tested, 37

At four different times while using this oil all the bees in the case became excited. They always produced an uproar by rapidly vibrating their wings.

Oil of thyme:

- 7 moved away quickly
- 6 moved antennae and then moved away slowly
- 4 moved away quickly and vibrated antennae
- 3 arose and moved away slowly
- 2 moved slightly and stroked antennae
- 2 arose quickly and vibrated antennae
- 1 jumped toward odor and stroked antennae
- 1 moved antennae quickly

Reaction time 2 to 3 seconds, average 2.4 seconds

Workers tested, 26

Oil of wintergreen:

- 11 moved antennae and moved away slowly
- 4 moved away quickly
- 3 moved away quickly and vibrated antennae
- 2 moved away slowly and stroked antennae
- 2 moved slightly and stroked antennae
- 2 moved away slowly and vibrated antennae
- 1 moved antennae slightly
- 1 turned around as if noticing odor

Reaction time 2 to 5 seconds, average 3.23 seconds

Workers tested, 26

Three times all bees in the case were excited

Honey and comb:

- 6 moved antennae and acted as if searching for the source of odor
- 6 stroked antennae several times
- 1 moved slightly as if noticing odor
- 4 vibrated antennae as if noticing odor
- 2 moved slightly and vibrated antennae
- 2 arose slightly and moved away slowly
- 2 vibrated antennae and put their heads on the floor above the vial
- 1 walked 2 inches toward odor, then turned around above the vial
- 1 arose slowly and acted as if searching for source of odor
- 1 raised antennae, turned around, and moved away slowly
- 1 turned around several times, stroked antennae, and acted as if searching for source of odor
- 1 arose quickly and held antennae on floor of case as if noticing odor

Reaction time 2 to 11 seconds, average 4.5 seconds

Workers tested, 31

Pollen:

- 10 vibrated antennae and acted as if noticing odor
- 3 turned around with their heads on the floor as if noticing odor
- 3 moved away slowly and stroked antennae
- 3 moved away slowly
- 3 stroked antennae and acted as if searching for source of odor
- 3 vibrated antennae and acted as if noticing odor
- 2 arose slightly and acted as if searching for source of odor
- 2 moved away quickly, then turned around as if noticing odor
- 2 arose and moved away slowly

Reaction time 2 to 16 seconds, average 5.37 seconds

Workers tested, 31

Flowers of honeysuckle:

- 6 vibrated antennae as if noticing odor
- 4 moved away quickly as if noticing odor
- 4 moved away quickly and vibrated antennae
- 3 turned around as if noticing odor
- 3 arose quickly and acted as if noticing odor
- 3 raised antennae and moved slightly
- 2 jumped slightly and acted as if noticing odor
- 2 stroked antennae and acted as if noticing odor
- 2 moved away slowly
- 1 moved to one side and acted as if searching for source of odor
- 1 vibrated antennae and walked an inch toward the vial

Reaction time 2 to 10 seconds, average 3.6 seconds

Workers tested, 31

Leaves and stems of pennyroyal:

- 6 moved away quickly
- 4 moved away slowly
- 4 stroked antennae and moved away slowly
- 3 vibrated antennae as if noticing odor
- 3 arose quickly and moved away quickly
- 2 moved their heads near floor as if noticing odor and moved away slowly
- 1 arose quickly and stroked antennae
- 1 jumped slightly and moved away quickly
- 1 vibrated antennae and turned around as if noticing odor

Reaction time 2 to 5 seconds, average 2.6 seconds

Workers tested, 25

Twice this odor excited all the bees in the case

Leaves and stems of spearmint:

- 10 moved away quickly
- 4 moved away slowly
- 3 turned around as if noticing odor
- 3 jumped quickly toward odor
- 2 vibrated antennae and moved away slowly
- 1 moved away slowly as if noticing odor
- 1 moved away quickly and stroked antennae

Reaction time 2 to 4 seconds, average 2.48 seconds

Workers tested, 24

Leaves and stems of scarlet sage:

- 10 moved away slowly
- 4 moved slightly and acted as if noticing odor
- 3 moved away quickly as if noticing odor
- 2 vibrated antennae as if noticing odor
- 2 moved to one side slowly and vibrated antennae
- 2 arose slowly, stroked antennae, and moved slightly
- 1 arose quickly
- 1 jumped slightly and moved away slowly

Reaction time 2 to 5 seconds, average 3.14 seconds

Workers tested, 25

Only once were all the bees in a case excited

Bee stings:

13 vibrated antennae and acted as if noticing odor

6 moved an inch or two until they were directly over the vial

3 stroked antennae and acted as if noticing odor

2 moved slightly and vibrated antennae

1 moved away quickly

1 turned around quickly as if noticing odor

1 jumped quickly and vibrated antennae as if noticing odor

1 moved quickly, vibrated antennae, and followed odor when vial was moved
beneath the case

Reaction time 2 to 3 seconds, average 2.16 seconds

Workers tested, 28

Smoke:

During all these experiments it was absolutely necessary to refrain from smoking tobacco because the least smoke in the laboratory invariably excited the bees. They produced an uproar by rapidly vibrating their wings, became very restless, and remained in this unquiet state for some time after being irritated. The least amount of smoke from a bee keeper's smoker, whether inside or near the outside of the house, also usually excited them.

Queens

Oil of peppermint:

3 moved away quickly

3 raised antennae and moved away slowly

2 moved away quickly and vibrated antennae

2 moved away slowly

1 vibrated antennae

1 arose and moved away

Reaction time 2 to 7 seconds, average 3.8 seconds

Queens tested, 12

Once all the workers in a case were excited

Oil of thyme:

3 stroked antennae and moved to one side

2 arose quickly and moved to one side slowly

2 moved away slowly and vibrated antennae

2 vibrated antennae

1 moved away slowly and vibrated antennae

1 lifted antennae and arose slowly

Reaction time 3 to 5 seconds, average 3.9 seconds

Queens tested, 11

Oil of wintergreen:

- 4 raised antennae and moved away slowly
- 2 moved away slowly and stroked antennae
- 2 moved away slowly and vibrated antennae
- 1 moved away slowly
- 1 vibrated antennae
- 1 arose and moved slightly

Reaction time 3 to 7 seconds, average 4.2 seconds

Queens tested, 11

Honey and comb:

- 4 vibrated antennae and moved away slowly
- 2 vibrated antennae
- 1 arose and moved slightly

Reaction time 5 to 10 seconds, average 6 seconds

Queens tested, 7

Pollen:

- 4 vibrated antennae and moved slightly
- 2 vibrated antennae
- 1 moved only slightly

Reaction time 4 to 10 seconds, average 6.3 seconds

Queens tested, 7

Leaves and stems of pennyroyal:

- 3 vibrated antennae
- 2 vibrated antennae and moved away slowly
- 1 vibrated antennae and turned around slowly
- 1 vibrated antennae and arose

Reaction time 3 to 8 seconds, average 5.1 seconds

Queens tested, 7

*Drones**Oil of peppermint:*

- 14 moved away quickly
- 3 raised antennae and moved away quickly
- 3 arose and moved away quickly
- 2 moved away slowly
- 2 moved away quickly and vibrated antennae
- 1 raised antennae quickly

Reaction time 2 to 3 seconds, average 2.3 seconds

Drones tested, 25

Only once were all the workers in a case excited

Oil of thyme:

- 14 moved away quickly
- 4 moved away slowly
- 3 moved slightly and vibrated antennae
- 3 arose slowly and moved away quickly
- 1 raised antennae

Reaction time 2 to 4 seconds, average 2.16 seconds

Drones tested, 25

All workers in the case were excited only once

Oil of wintergreen:

12 moved away quickly

6 arose quickly and moved away slowly

3 arose, stroked antennae, and moved away slowly

2 raised antennae quickly and moved away slowly

2 moved antennae slightly

Reaction time 2 to 4 seconds, average 2.48 seconds

Drones tested, 25

Honey and comb:

5 moved antennae slightly

4 vibrated antennae vigorously

2 raised antennae and turned around as if noticing odor

2 vibrated antennae vigorously and tried to reach the honey through the floor of the case

2 vibrated antennae as if noticing odor

2 arose slowly and vibrated antennae

1 placed antennae on floor of case and acted as if noticing odor

1 stroked antennae and turned around as if searching for honey

Reaction time 2 to 10 seconds, average 3.8 seconds

Drones tested, 19

Pollen:

5 vibrated antennae and moved slightly

2 vibrated antennae vigorously

2 vibrated antennae and acted as if noticing odor

2 raised antennae and moved away slowly

2 arose quickly and stroked antennae

2 arose and moved away slowly

1 tried to get through the floor of the case

1 moved only slightly

Reaction time 2 to 6 seconds, average 3.56 seconds

Drones tested, 17

Flowers of honeysuckle:

5 moved away quickly

4 moved away slowly

3 vibrated and stroked antennae

3 vibrated antennae as if noticing odor

3 arose quickly and moved away slowly

1 raised antennae quickly and moved away quickly

1 arose quickly and turned around as if noticing odor

Reaction time 2 to 5 seconds, average 2.8 seconds

Drones tested, 20

Leaves and stems of pennyroyal:

- 8 moved away quickly
- 6 raised antennae and moved away slowly
- 3 moved away slowly
- 3 vibrated antennae vigorously
- 2 arose and moved away slowly
- 2 arose slowly
- 1 arose quickly and stroked antennae

Reaction time 2 to 5 seconds, average 2.74 seconds

Drones tested, 25

Leaves and stems of spearmint:

- 6 moved away quickly
- 5 vibrated antennae and moved slightly
- 4 arose and moved away slowly
- 3 moved away slowly
- 1 raised antennae and moved slightly

Reaction time 2 to 4 seconds, average 2.55 seconds

Drones tested, 19

Once all the workers in a case were excited

Leaves and stems of scarlet sage:

- 4 arose quickly and moved away slowly
- 4 vibrated antennae slightly
- 3 moved away slowly
- 3 raised antennae and moved away slowly
- 2 vibrated and stroked antennae and moved away slowly

Reaction time 2 to 10 seconds, average 3.37 seconds

Drones tested, 16

Summary

To summarize the preceding data, it is found that 87 per cent of the individuals tested moved away from the peppermint, 80 per cent from thyme, 89 per cent from wintergreen, 6 per cent from honey, 20 per cent from pollen, 45 per cent from honeysuckle, 78 per cent from pennyroyal, 73 per cent from spearmint, 69 per cent from scarlet sage, and 4 per cent from the bee stings. Thus, to all of these odors, except those of honey, pollen, honeysuckle, and the bee stings, bees react negatively. For the odors of honey, pollen, and honeysuckle the most characteristic response is in searching for the source of the odor by turning around over the vial with the head almost on the floor of the case and by vibrating the antennae vigorously. In response to the bee-

sting odor the bees usually moved quickly toward the source of the odor when the vial was moved 2 or 3 inches from them. By including the data from the use of all of these ten odors, except that of bee stings, the average reaction time for the very first responses of all the workers is 3.29 seconds, whereas the average reaction time for all the drones is 2.86 seconds. By using the data for the first responses to peppermint, thyme, wintergreen, honey, pollen, and pennyroyal, the average reaction time for all workers is 3.4 seconds, for all drones 2.9 seconds, and for all queens 4.9 seconds. Hence, in spite of the fact that drones are less active in the observation cases, they responded somewhat more quickly than did the workers and much more quickly than did the queens.

It is evident, therefore, from these data that the olfactory sense in the honey bee is acute and that the sensitiveness to various odors is most highly developed in the drones, and least highly developed in the queens.

EXPERIMENTS ON MUTILATED BEES IN OBSERVATION CASES

In the preceding pages the behavior of unmutilated bees in observation cases and their reactions to odors are discussed. The behavior of bees that have been injured in various ways will now be discussed, together with an account of the experiments upon them with various odors. In these experiments all the bees in the same observation case were mutilated and they were given the same kinds of food as those on which the uninjured bees survived.

ANTENNAE

Entomologists now generally agree in the belief that the organs of smell in insects are located on the antennae. Bees with either the right or left antenna pulled off are much less pugnacious than are those with the antennae intact, and they "pay less attention" to each other. They appear otherwise normal, except that their ability to communicate is considerably decreased. They feed the queen and each other, eat normally, and often stroke each

other as do those with both antennae intact. While those with both antennae intact lived on an average of 9 days and 3 hours in observation cases, the bees with one antenna removed lived under the same conditions on an average only 6 days and 18 hours.

Bees with one antenna pulled off and with 2 to 8 joints of the other one cut off never "pay any attention" to each other and very seldom are seen fighting, but are just as apt to fight a hive-mate as a stranger. All of these eat more or less, but the more joints cut off the second antenna the less normal they appear. The greater the number of joints severed, the less number of days they live. Such bees, on an average, lived 5 days and 11 hours in the observation cases.

Bees with both antennae pulled off at first wander about inside the case more or less aimlessly. They seem to have no means of communicating with other bees. They run against each other and then try either to stroke or to fight one another, although they fail to do either. Only occasionally do they feed one another and only a few are seen eating. After a few hours they become inactive and usually remain so until they die. In their activity they are similar to young bees. They "pay little or no attention" to other bees and in most cases do not move unless another bee runs against them. The ones studied lived on an average of only 19 hours in the observation cases.

Bees with both antennae cut off at their bases behave very similarly to those just described. Those observed lived only 2 hours on an average in the observation cases.

When the antennae were varnished with shellac or celloidin the bees were not at all normal. They failed to eat; many ran around and acted "crazy" and tried to clean their antennae, but in this they failed. Those with shellac on their antennae lived only a few hours, while those with celloidin lived, on an average, 16 hours in the observation cases.

The antennae of some were covered with vaseline. These bees at once cleaned their antennae and were soon normal.

The left antennae of 15 workers were pulled off at the base with a small pair of forceps. The responses of these bees to the

odors of the three essential oils are similar to those described for bees with both antennae intact. The average reaction times are as follows: Oil of peppermint 5.2 seconds, oil of thyme 4.3 seconds, oil of wintergreen 4.8 seconds. The average time for 15 specimens with their right antennae pulled off gave similar results, as follows: Oil of peppermint 4.8 seconds, oil of thyme 4.1 seconds, oil of wintergreen 4.6 seconds. The average time for workers with one antenna pulled off is: Oil of peppermint 5 seconds, oil of thyme 4.2 seconds, oil of wintergreen 4.7 seconds. This gives a general average of 4.6 seconds for the three oils, while the general average of the same oils for bees with both antennae intact is only half as much, that is, 2.3 seconds. According to these results, and on the theory that the antennae contain the organs of smell, it would at first glance seem that each antenna plays an equal part in receiving odor stimuli and that when either antenna is absent the average time for the responses is consequently doubled.

Bees with either the right or left antenna pulled off and with from 2 to 8 joints of the remaining flagellum cut off gave the following average reaction times when using the three essential oils:

	<i>seconds</i>
2 joints missing	15
4 joints missing.	44
5 joints missing	56
6 joints missing.	27
7 joints missing.	98
8 joints missing.	88

Thus, in general, the greater the number of joints of the second antenna removed the longer it takes the bees to respond to odor stimuli. Workers thus mutilated are not at all normal and live on an average only 5 days and 11 hours.

Bees with both antennae either pulled off or covered with celloidin entirely fail to respond to the essential oils. They also are quite abnormal in their behavior.

Two drones, one of which had 4 and the other 5 joints of one flagellum missing, were taken in this condition from a hive.

These were apparently normal in other respects, but when tested with 6 of the 10 odors used in these experiments they gave an average reaction time of 3.16 seconds, whereas the average for the same odors with un mutilated drones was 2.9 seconds.

Ninety middle-aged bees with both antennae pulled off were placed on the combs of the observation hive at 4 o'clock in the afternoon. To prevent them from flying from the hive, their right wings were clipped. These bees were more restless than normal bees. They wandered about and continually crawled out of the hive. Whenever one was found outside the hive it was put back, although all of them eventually escaped in this way, because two days later not one of them, either alive or dead, was found in the hive. The behavior of these mutilated bees was similar to that of the antenna-less bees in the observation cases. They took no part in the activities of the hive and appeared to have lost all means of communicating with the other bees. The next day at various hours 16 of them were found dead in front of the hive. The third day at 8 o'clock 3 more were found, and on the fourth day at 4 o'clock one more. In three days 20 were found dead, while the other 70 certainly crawled out of the hive and escaped. Counting the time until their dead bodies were found, these 20 lived on an average only 21 hours, while in the observation cases the average length of life of such bees was 19 hours. From these combined results it is evident that bees with their antennae pulled off are not normal and therefore whatever results are obtained by experimenting with them must always be discounted.

Immediately after pulling off both antennae of a worker, it was placed on a comb with the other bees in the observation hive. A small piece of cotton wet with oil of peppermint was held $\frac{1}{2}$ inch in front of this antenna-less bee, and afterward smoke from a bee keeper's smoker was gently blown on it. In performing these experiments with 10 different workers, not a single mutilated bee reacted in the least, whereas all the other bees soon fled from the oil of peppermint and caused an uproar by rapidly vibrating their wings when smoke was gently blown on them. Similar experiments were performed, by using oil of thyme, clove, wintergreen,

and cedar and the same bees were afterwards tested with smoke. Not one of 50 mutilated bees so treated showed any reaction, while the normal bees never failed to react.

The mutilated bees in these experiments were restless and wandered about considerably. They often crawled into cells. Occasionally one cleaned itself and sometimes other workers cleaned them; very often a mutilated worker fed one to three other bees before it crawled off the comb and disappeared. How long these mutilated bees lived can not be stated because they were soon lost among the other bees. Three or four hours afterward two or three of them were found lifeless in front of the hive.

Miss Fielde ('03 a) believes that the olfactory organs which an ant uses in recognizing enemies lie in the fifth and sixth antennal segments. To ascertain if one or more particular antennal joints of bees bear olfactory organs which are used in recognizing strange bees, 3 to 8 joints were cut from both antennae of 3 lots of 9 middle-aged bees each. These mutilated workers were introduced into an observation case and their behavior was studied. No abnormality in behavior was noticed among them except that they were slightly less active. Occasionally when first introduced one attacked another, although not seriously, and no injury was ever done to any of them. They lived scarcely 9 days on an average.

Nine bees with 2 to 8 joints of both antennae amputated were introduced into an observation case. The following day several strange bees, one at a time, were put into the same case. Most of the mutilated bees at once noticed a stranger and in a few seconds one of the strange bees was attacked. Both fighting bees were immediately removed from the case, the stranger was discarded, while the mutilated bee was killed and its remaining antennal joints counted. This experiment was repeated seven times. In all, 72 mutilated bees and as many uninjured ones were used. In only a few instances did the mutilated bees attack each other. This can always be prevented by keeping the bees isolated for a short time after the operation. When strange bees were put among the mutilated ones almost one-half of the

former were attacked. When the remaining antennal joints were counted it was found that in the pugnacious bees from 2 to 6 antennal joints were missing and in those that did not attack the strangers from 2 to 8 joints were absent. Only the last 8 joints of the antennae of a worker contain the supposed olfactory organs and when these 8 joints were removed all of these organs are eliminated. According to Miss Fielde's theory the fifth and sixth antennal segments of the worker bee would be the ones which carry the olfactory organs by which strange bees are recognized. Furthermore, Miss Fielde claims that the tenth antennal joint of an ant contains the organs through which the colony odor is recognized and if this joint is removed colony-mates will attack each other. Judging from the foregoing experiments, no particular antennal joint of a worker bee contains the organs by which the odor of sister bees is received, because the mutilated bees never fought each other regardless of the number of antennal segments amputated, when they were kept out of the cases for a few minutes after the antennae were injured.

To determine whether bees with mutilated antennae are normal, 11 joints of both antennae of 12 middle-aged bees were cut off. When introduced into an observation case these bees did not fight, "paid no attention" to each other, were quiet, and only one ate candy. When strange bees were put among them the mutilated bees always gave the strangers the right-of-way and did not attack them. They lived only 6 hours on an average.

Both antennae of 95 middle-aged bees were burnt off with a red-hot needle. These workers were also abnormal and lived only 7 hours on an average. The antennae of 30 drones were similarly burnt off and they too lived only 17 hours on an average.

Thirty middle-aged bees were immersed in water for 15 minutes. When removed they appeared entirely lifeless and their antennae were pulled off at once. They revived and lived only 19 hours on an average.

Since bees whose antennae are mutilated after they become adults are abnormal, the following experiments were performed with immature bees. A frame of brood was removed from a hive, the cells of sealed brood were gently uncapped and both

antennae of 100 pupae ranging in age from 14 to 18 days from the laying of the egg were carefully cut off near the head. Great care was exercised not to injure the immature bee in any way other than by the amputation of these appendages. The cells were again closed with a very thin layer of beeswax, a wire-cage screen was placed over these uncapped cells so that the adult workers could not pull the mutilated bees out of their cells, and the frame was put into the hive. In a few days these bees began to emerge from their cells. They were removed from the hive and placed in observation cages with intact middle-aged bees. All bees thus mutilated were abnormal and lived as adults about 5 days on an average.

In like manner both antennae were cut from 300 pupae 13 and 14 days old from the laying of the egg. In this instance a wire screen was placed over the entire frame so that the mutilated bees could not escape when they emerged. Seven days later they began to emerge and most of them in a short time crawled from their cells. They soon mixed on this comb with other young bees that were unamputated and they had plenty of food but they were all dead 5 days later.

Seven of the workers with their antennae burned off recovered from the operation sufficiently to eat candy and to move about freely, but they were far from being normal. These lived from 1 to 11 days. When tested with the three essential oils, peppermint, thyme, and wintergreen, they responded readily. Their general response was to move slightly and vibrate the stubs of their antennae; one rubbed a leg against the abdomen and 3 moved their heads quickly. Average reaction time for oil of peppermint 3.5 seconds, for oil of thyme 4.5 seconds, for oil of wintergreen 4.3 seconds, and for all three oils 4 seconds. Frequently, when the antennae were cut or burnt off, the insects were placed on the table and tested with these odors. They often moved away from the odors but generally did not react to odors or anything else and often scarcely moved even when touched with a pencil.

In previous experiments bees cleaned off any substance put on their antennae. To prevent this the tarsi of their front legs

were burned off with a red-hot needle, removing their antennal cleaners. About one-fourth of the bees so mutilated died within 12 hours, but the remainder appeared quite normal in every other way. On the second day eleven joints or the entire flagellum of each antenna was coated with liquid glue. Since the antennal cleaners were removed, these bees could not remove the thick coating of glue. They were quite abnormal and most of them did not live long. However, after gluing the flagella of many bees, 21 were finally obtained that were fairly normal and they responded to the odors of the above oils without failure. The general response was either to arise and move away quickly or to vibrate their antennae. Often when one had fallen down apparently lifeless the odor was placed under it; it arose almost instantaneously and moved away quickly. After 3 or 4 days all the surviving bees had succeeded in cleaning their antennae but most of them died before this time. However, some lived 12 to 14 days. The average reaction time to oil of peppermint was 3.1 seconds, to oil of thyme 3 seconds, to oil of wintergreen 2.8 seconds, and for all three oils 2.9 seconds.

To ascertain if the odor of the glue itself affected these results in any way, glue was placed on the top of the thorax. This dried in a few minutes and the bees were not able to get it off. They were entirely normal and were tested with the foregoing odors. The average reaction time to oil of peppermint was 2.4 seconds, to oil of thyme 2.8 seconds, to oil of wintergreen 3.1 seconds, and for all three oils 2.7 seconds. The workers tested were 19 in number.

From all the experiments performed on the antennae of bees it is evident that when these appendages are mutilated in the slightest degree, the bees are never entirely normal, even though they apparently recover from the effect of the shock. The greater the number of joints removed or covered, the greater the abnormality. Furthermore, there is no reason to assume the presence of the olfactory organs on the antennae, because when these appendages were burned off, the general average of the reaction time for the three oils was 4 seconds; when glued, 2.9 seconds; and when un mutilated, 2.6 seconds. This slight in-

crease of time can certainly be attributed to the abnormal condition of the bees, and these results indicate that the olfactory organs are located elsewhere. At most it can be claimed only that the antennae may assist in the receiving of odor stimuli.

MAXILLAE AND LABIAL PALPI

Various observers claim that the palpi of insects are the seat of the olfactory organs. In the bee the maxillary palpi are almost wanting, but the maxillae and labial palpi possess pore-like sense organs. These appendages were cut off at the base. When introduced into observation cases, bees so treated appeared quite normal in all other respects and when strange bees were put among them they lost no time in attacking the strangers. Nevertheless, they certainly were not completely normal, for they lived only from 3 hours to 4 days, with 24 hours as an average. When tested with odors, they gave responses similar to those of uninjured bees. The reaction time was as follows: Oil of peppermint 2.2 seconds, oil of thyme 3.7 seconds, oil of wintergreen 4 seconds, honey and comb 4.4 seconds, pollen 5.8 seconds, and leaves and stems of pennyroyal 3.9 seconds. This gives a general average of 4 seconds, whereas for the same odors with un-mutilated bees the general average was 3.4 seconds.

PROBOSCIS

Several writers have described sense organs on the proboscis of insects. To determine whether these have an olfactory use, the proboscides of 36 workers were cut off close to the base. These bees seemed quite normal in most respects and many of them even tried to eat candy, but of course they could not accomplish much without this appendage. They lived only 7 hours on an average. The average reaction time to oil of peppermint was 2.6 seconds, to oil of thyme 3 seconds, and to oil of wintergreen 3.2 seconds. This gives a general average of 2.9 seconds, while for the same odors with un-mutilated bees the average was 2.6 seconds. Twenty-two workers were tested. We can probably attribute this difference of 0.3 seconds to the abnormality of the mutilated bees.

MANDIBLES

Janet ('11b) describes a sense organ in the mandible of the honey bee which he thinks may have an olfactory function. To ascertain this experimentally, the mandibles of 30 workers were amputated close to the base. These bees appeared completely normal and did not fight each other. They lived from 5 hours to 15 days, with 7 days as an average. The average reaction time to oil of peppermint was 2.6 seconds, to oil of thyme 3.3 seconds, to oil of wintergreen 4.6 seconds, to honey and comb 7.7 seconds, to pollen 6.2 seconds and to leaves and stems of pennyroyal 4.7 seconds. These give a general average of 4.8 seconds whereas the average for the same odors with unmutilated bees was 3.4 seconds. We may attribute this slight difference in time either to the injury caused by the amputation, or to the fact that the mandibles help to perceive odors or to both. The bees tested were 20 in number.

BUCCAL CAVITY

Huber (1814) states that the seat of the organs of smell in the honey bee is the buccal cavity, whereas Wolff (1875) discovered some sense organs on the epipharynx of the same insect. The epipharynx lies in the mouth cavity and forms a portion of its roof. To determine whether this cavity has anything to do with olfaction, Huber's experiment of filling the mouth cavity with flour paste was repeated. With the aid of a small pencil brush the mouth cavities of 25 workers were thus filled. When the paste had become perfectly dry, the bees were put into observation cases. They seemed otherwise entirely normal and all tried to eat candy although some were unable to move their probosces on account of the hard paste. They lived from 2 hours to 16 days, with $7\frac{1}{2}$ days as an average. However, by the end of the fourth day the paste had come out of their mouths. The average reaction time to oil of peppermint was 2.55 seconds, to oil of thyme 2.75 seconds, and to oil of wintergreen 2.75 seconds. This gives a general average 2.68 seconds. The average for

the same odors with normal bees was 2.64 seconds. Twenty workers were tested. It would seem that neither the buccal cavity nor the epipharynx has anything to do with olfaction.

MORPHOLOGY OF OLFACTORY PORES

In 1857 to 1860 Hicks described for the first time some peculiar structures found on the bases of the wings and legs of insects. He called them 'vesicles' and suggested that they have an olfactory function. From the following description of the morphology of these structures it will be seen that the word 'vesicle' is less appropriate than the word 'pore.' Since they have an olfactory function in the honey bee (pp. 333-341) they may be called 'olfactory pores.'

DISPOSITION

In studying the distribution and number of the olfactory pores of the honey bee, workers and drones just emerged from the cells were selected on account of their lighter color, but since queens at this stage were not available at the right time, old dark-pigmented ones were used. The three legs from one side of 6 individuals each of workers, queens, and drones and the three legs from the other side of 9 of these 18 bees were examined under a high-power lens. The wings from both sides of 8 workers, 8 drones, and 4 queens and the stings of 15 workers and of 9 queens were likewise examined. In all, 81 legs, 80 wings, and 24 stings were searched for pores.

The wings have upper (dorsal) and lower (ventral) surfaces and the legs have inner and outer surfaces. The inner side of the leg faces the bee's body, and the outer side is directed from the body except in the case of the front legs, which are directed forward and the sides are therefore reversed. These pores are found in groups, and for convenience in studying and describing them the groups on each side may be numbered from 1 to 21. The first five of these groups are found at the bases of the wings, groups 6 to 18 inclusive on the legs, and the remaining three groups on the sting (figs. 1, 2, and 14, A). The numbers of the

ABBREVIATIONS

- IA*, 3*A*, first anal vein, third anal vein
AgCyl, deeply staining cytoplasm
Art, articulation
ArtCh, articular chitin
ArtM, articulation membrane
1Ax, 2*Ax*, 3*Ax*, first axillary, second axillary, third axillary
AxC, axillary cord
BC, body cavity
BGL, alkaline gland of sting
BICor, blood corpuscle
BlSin, blood sinus
Brb, barb
BrbHr, barbed hair
C, costa
Ch, chitin
ChL, chitinous layer
ChM, chitinous marking
ChTh, chitin of thorax
Con, cone
ConnT, connective tissue
Cu, cubitus
Cyl, cytoplasm
FFL, Forel's flask or sensilla ampullacea
Gv, groove
Hr, hair
Hyp, hypodermis
HypCW, hypodermal cell wall
HypNuc, hypodermal nucleus
HypNuc¹, hypodermal nucleus that has formed a hair
IXS, thick membranous lobe that overlaps sting
Lct, lancet
M, muscle
MB, muscle bundle
MBNuc, nucleus of muscle bundle
Md, median plate
MFl, mouth of flask
N, nerve
NB, nerve branch
Neu, neurilemma
NeuNuc, nucleus of neurilemma
NeurNuc, neuroglia nucleus
NF, nerve fiber
NkFL, neck of flask
Ob, oblong plate of sting
Pg, peg, club, or sensilla basiconica
Por, pore
PorAp, pore aperture
PorApHr, pore aperture of hair
PorB, pore border
PorBHr, pore border of hair
PorCl, pore canal
PorHr, pore of hair
PorPl, pore plate, pore canal, or sensilla placodea
PorW, pore wall
PorWHr, pore wall of hair
PPg, pit pegs, champagne-cork organs or sensilla coeloconica
PsnCl, poison canal of sting
PsnSc, acid poison sac of sting
Qd, quadrate plate of sting
R, radius
R+M, radius and media united
Sar, sarcolemma
SarNuc, nucleus of sarcolemma
SC, sense cell
SCG, sense cell ganglion
ScL, scutellum of mesotergum
SCNuc, nucleus of sense cell
SCNuc¹, nucleus of sense cell without cell wall
SCNucl, nucleolus of sense cell
SF, sense fiber
Sh, shaft of sting
ShB, bulb of shaft of sting
SpHr¹, 2, 3, three varieties of spinelike hairs
StnPlp, palpus of sting
1Tar, 2*Tar*, 3*Tar*, 4*Tar*, 5*Tar*, first to fifth tarsal joints
Tg, tegula
THr, tactile hair or sensilla trichodea
Tn, taenidia of trachea
Tra, trachea
TraNuc, nucleus of trachea
Tri, triangular plate of sting
1 to 21, groups 1 to 21 of the olfactory pores

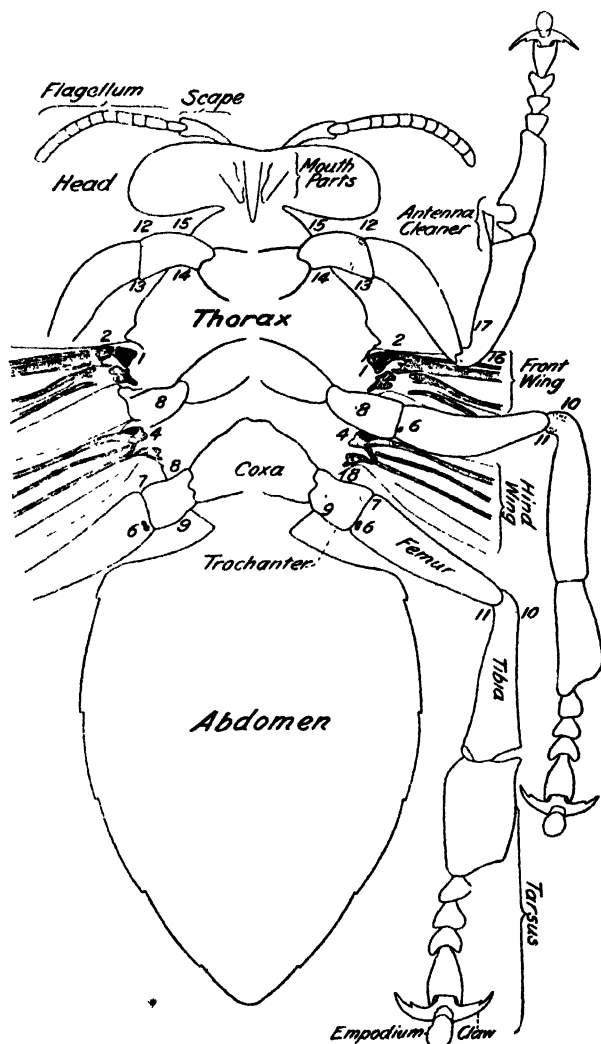


Fig. 1 Diagram of ventral view of a worker bee, showing the location of the different groups of olfactory pores as indicated by the numbers:

groups on the legs apply to similarly placed groups on all the legs, but the groups on the front and hind wings are seemingly not homodynamous and are given different numbers. If a group has been found to be always similarly placed and always

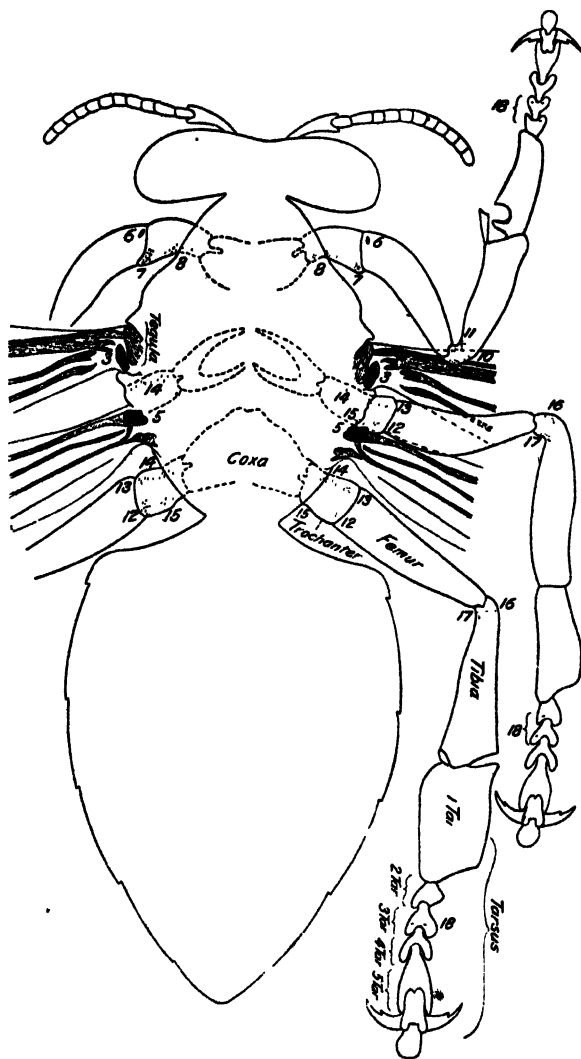


Fig. 2 Diagram of dorsal view of a worker bee, showing the location of the different groups of olfactory pores as indicated by the numbers.

to have the same general shape, it may be regarded as constant and if the pores are close together it may be regarded as definite; otherwise it may be considered as inconstant and scattered.

As the worker bee is most suitable for study, the pores on the wings, the third leg, and the sting of specimen No. 15, which has been drawn (figs. 1 and 2), will be described in detail. The differences on the other legs of this specimen and the variations found in the three castes will then be discussed.

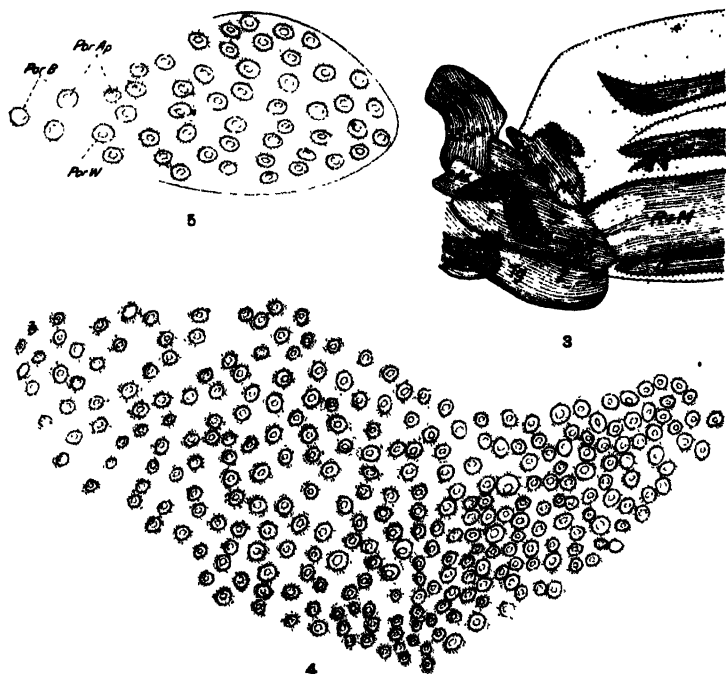


Fig. 3 Ventral view of base of front wing of a worker bee, showing groups 1 and 2 of olfactory pores. $\times 45$.

Fig. 4 External view of group 1 of olfactory pores of a worker bee. $\times 465$.

Fig. 5 External view of group 2 of olfactory pores of a worker bee. $\times 465$.

Groups 1 and 2 (figs. 1 and 3) lie on the lower surface of the front wing. Group 1 occupies almost a central position on the subcosta, while group 2 lies near the anterior and distal margin (fig. 3, *Sc.*). These groups are somewhat triangular in shape, with their apices facing each other, and the concave edge of group 1 always faces the posterior margin of the subcosta. A light band (represented by a line in fig. 5), surrounds the distal

end of group 2. In groups 1 and 2 there are 281 and 50 pores respectively; in the former group (fig. 4) the pores are close together while in the latter (fig. 5) they are more scattered. The pores vary considerably in size; in group 1 most of them are small, with the diameter of the largest three times that of the smallest, while in group 2 almost all of them are comparatively large, with the diameter of the smallest one-half that of the largest. In group 1 scarcely any rows are discernible, but in group 2 the rows are more sharply defined. The openings (*PorAp*) in the pores are usually round, although several are oblong, and the long diameter of the oblong pores is more or less parallel with that of the group.

Group 3 is found on the upper surface of the front wing (fig. 2) and occupies nearly the entire surface of the median plate (fig. 6, *Md*), leaving only a narrow margin on all sides. This group is long and slender, with its tapering end pointing toward the proximal and posterior margin of the subcosta (*Sc*). Its pores are considerably scattered, although they lie in more or less definite rows (fig. 7). The pores are about equal in diameter and the diameter of the largest is never more than twice that of the smallest. In this group there are 174 pores, nearly all of which have round apertures.

Group 4 is present on the lower surface of the hind wing (fig. 1) and covers most of the anterior half of the union of the radius and media (fig. 8, *R + M*). Its distal end is the more pointed and its pores are arranged in irregular rows (fig. 9). There are 83 pores of about equal size.

Group 5 lies on the upper surface of the hind wing (fig. 2) and occupies nearly all of the surface where the radius and media unite (fig. 10, *R + M*) and extends slightly over the subcosta (*Sc*). The proximal and narrower end of the group points directly toward the proximal end of the subcosta. There are 209 pores, which can scarcely be said to lie in rows (fig. 11). Most of the pores are small, with the diameter of the largest three times that of the smallest.

Group 6 is a double group having the shape of a figure 8, located at the proximal end of the femur (fig. 1) on the outer

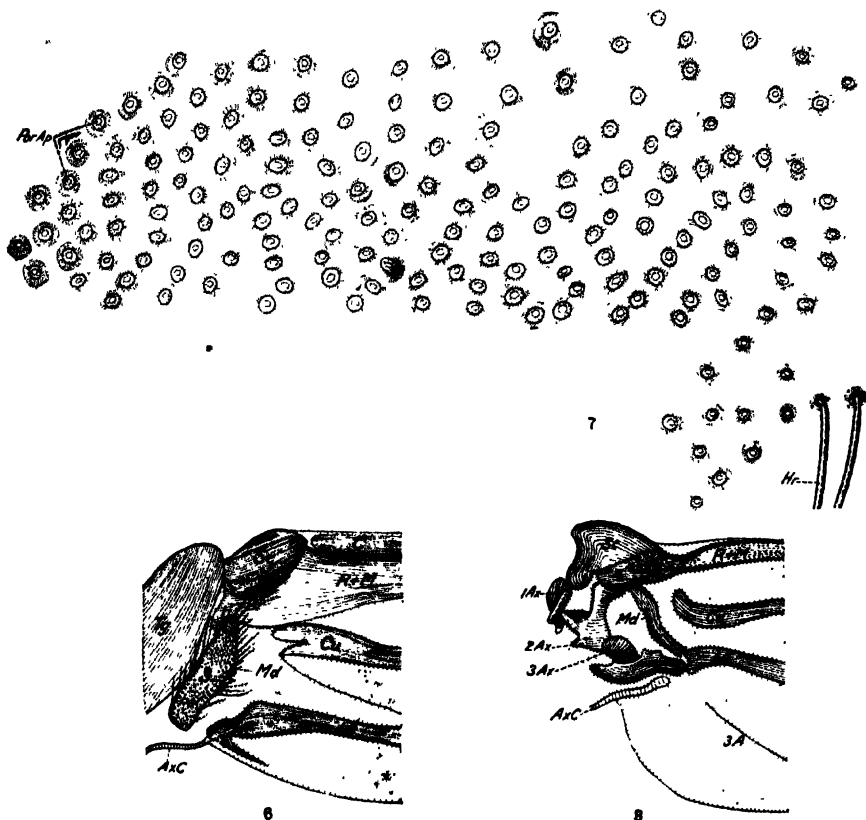


Fig. 6 Dorsal view of base of front wing of a worker bee, showing group 3 of olfactory pores. $\times 45$.

Fig. 7 External view of group 3 of olfactory pores of a worker bee. The distal end with two hairs is placed beneath the remainder of the group. $\times 465$.

Fig. 8 Ventral view of base of hind wing of a worker bee, showing group 4 of olfactory pores. $\times 45$.

surface near the posterior margin. The smaller portion of group 6 lies nearer the posterior edge of the leg (fig. 12, A) and has 9 pores, none of whose openings lies with its long diameter exactly transverse to the long axis of the leg. The other portion of the group has thirteen pores, and the long diameter of most of its openings are exactly transverse to the long axis of the leg. The diameter of the largest pore is about twice that of the smallest.

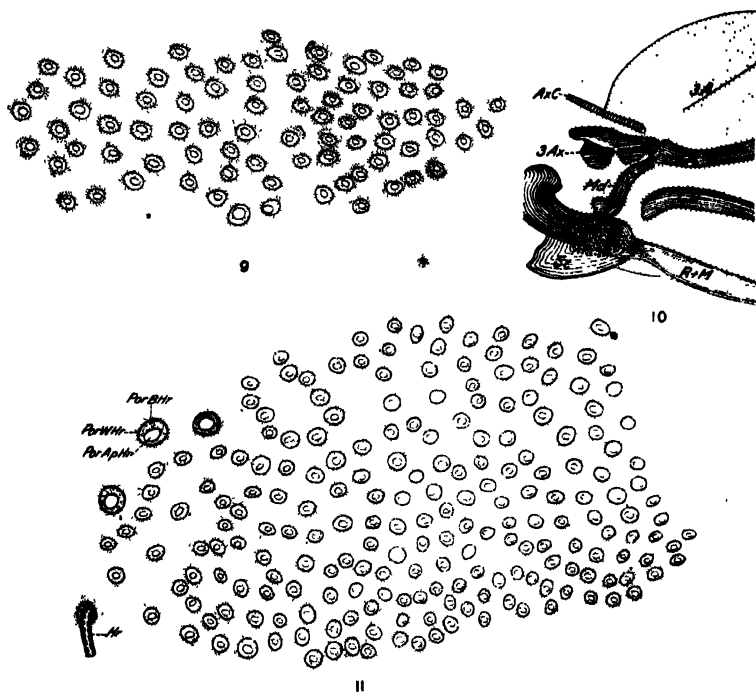


Fig. 9 External view of group 4 of olfactory pores of a worker bee. $\times 465$.

Fig. 10 Dorsal view of base of hind wing of a worker bee, showing group 5 of olfactory pores. $\times 45$.

Fig. 11 External view of group 5 of olfactory pores of a worker bee. $\times 465$.

The majority of the pores on the legs are slightly oblong and have openings which as a rule have their long diameters more or less transverse to the long axis of the leg.

Groups 7, 8, and 9 lie on the outer side of the trochanter (fig. 1). Group 7 has 16 pores and lies at the distal end near the anterior margin, group 8 extends along the anterior margin from group 7 almost to the articulation of the trochanter with the coxa, and group 9 extends from the proximal end along the median line two-thirds the distance to the femur. In all three groups the diameter of the largest pore may be three times that of the smallest. The pores of groups 7 and 9 (figs. 12, B and D) have their long diameters directed obliquely across the leg, while in

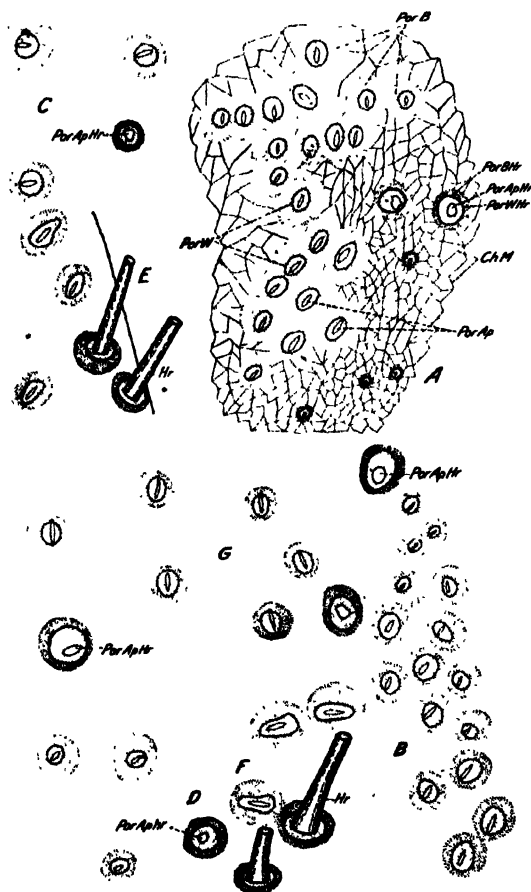


Fig. 12 External view of olfactory pores as they appear on the third leg of a worker bee, $\times 465$. A is group 6; B, group 7; C, three olfactory pores and a hair pore (*PorAphr*) from group 8; D, three olfactory pores and a hair pore from group 9; E and F, a few olfactory pores and hairs from groups 10 and 11; G, six olfactory pores and a hair pore from group 12.

group 8 (fig. 12, C) most of the long diameters are parallel to the long axis of the trochanter.

Groups 10 and 11 are found at the proximal end of the tibia (fig. 1) on the outer side. Group 10 is near the anterior edge and group 11 near the posterior. The pores of these groups are

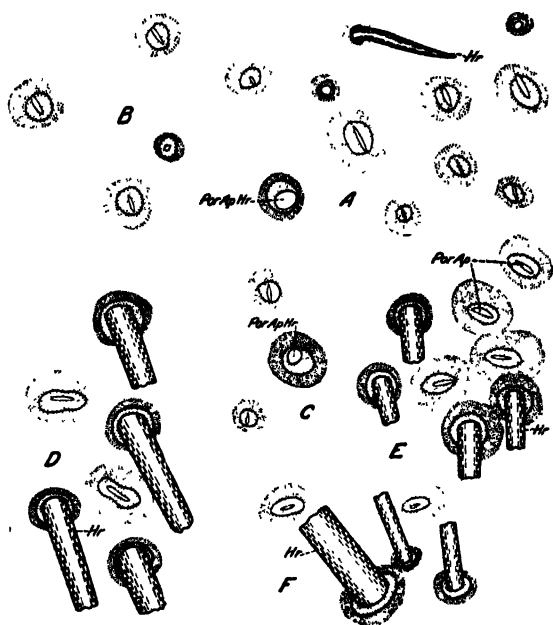


Fig. 13 External view of olfactory pores and some spinelike hairs as they appear on the third leg of a worker bee, $\times 465$. A, from group 13; B, from group 14; C, from group 15; D and E from groups 16 and 17 respectively; F, from group 18, the two pores are drawn twice too close together.

the largest of all and they do not vary greatly in size. They are oblong and the long diameters are in some cases oblique and in others transverse to the long axis of the tibia (fig. 12, E and F). They lie in rather straight rows, along the long axis of the leg.

Groups 12 to 15 lie on the inner side of the trochanter (fig. 2). Groups 12 and 13 are located at the distal end, with group 12 near the posterior edge and group 13 near the anterior. Group 14 lies on the inner side in a position near the anterior edge similar to that of group 8 on the outer side. Group 15 lies near the posterior margin extending the full length of the trochanter (figs. 12, G and 13, C). In these preparations occasionally one end of the slits extends outside the pore wall (*PorApH*). This may be due to the treatment with caustic potash.

Groups 16 and 17 (figs. 2, 13, D and E) are similar to groups 10 and 11 except that they are on the inner side of the proximal end of the tibia.

Group 18 consists of two or three pores as large as those on the tibia, which are found on the ventral side of the second and third tarsal joints (fig. 2). These two pores and three hairs are represented in figure 13, F, although in the drawing the relative distance between the pores is reduced one-half.

Group 19 is located on the shaft of the sting (fig. 14, A) and its pores are greatly scattered, but most of them occur about midway between the two ends. A few are present on the tip end of the shaft (fig. 14, A, *a*), while three are located at the other extreme end (fig. 14, A, *b*). This group contains 44 pores. Figure 14, B, shows the pores on the tip of the shaft with a high magnification. The canal (*PorCl*) passes from the apertures into the body cavity (*BC*) of the shaft. Snodgrass ('10) presents a similar drawing, although he represents too many pores near the tip end of the shaft. Figure 14, C, represents a pore from the tip end of the shaft at *a* and figure 14, D, two pores from the point marked *e* in figure 14, A, with a still higher magnification. Figure 14, E, represents five pores from the point marked *c*.

Group 20 occurs on the tip end of each lancet (fig. 14, A, *Brb*). Here there are 10 pores and an equal number of barbs, the opening of each pore being found at the base of a barb. With the highest magnification it is plainly seen that the canal of the pore runs into the body cavity of the lancet (fig. 14, F, *BC*).

Group 21 lies on each lancet just behind the lowermost part of the bulb of the shaft (fig. 14, A, *d*). It has 10 pores,² which vary considerably in size (fig. 14, G).

The following differences were found in the various groups of pores on the first and second legs of specimen No. 15: On each of these two legs group 6 is a single group and has only 18 pores. It does not lie quite so close to the posterior edge as on the third leg. Group 7 is not found on the second leg, but on the first it

² The drawing (fig. 14, G) shows only eight of these pores.

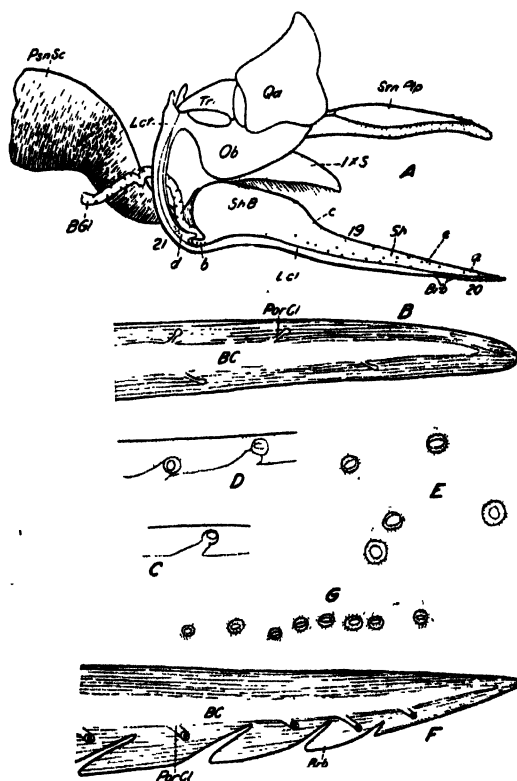


Fig. 14 Olfactory pores on the sting of a worker bee. A, diagram of lateral view of a worker bee's sting and its accessory parts, showing location of olfactory pores as indicated by the numbers; B, dorsal view of the tip end of shaft (*Sh*, fig. 14 A), showing the pore canals (*PorCl*) of olfactory pores running into the body cavity (*BC*), $\times 185$; C and D represent pores from *a* and *e* of figure 14 A, respectively, $\times 465$; E shows five pores from *c* of 14 A, $\times 465$; F, tip end of lancet, showing pore canals (*PorCl*) running into the body cavity (*BC*), $\times 465$; G, eight of the ten pores from group 21, $\times 465$.

consists of 15 pores. Group 8 is constant in position on all three legs, but group 9 is not found on the first two. Groups 10 and 11, 16 and 17 are constant, with practically the same number of pores on each leg. Groups 12 to 15 are present on all three legs but may vary slightly in position and do vary greatly in number of pores. On the second leg group 12 is definite, with 15 pores. Group 18 is constant, consisting of 2 or 3 pores.

Thus, for specimen No. 15, groups 1 to 6 are strictly definite and constant. Group 7 is not always present, but it is definite when found. Group 8 and groups 10 to 18 are always present, and are scattered, groups 8, 14, and 15 being scattered most, and they may vary slightly in position. Group 9 is found only on the third leg, and it is scattered more than any other group.

Variation

The groups of pores differ in shape and size in the different legs of the same individual, and different individuals show slight variation in homologous groups. The following account gives the variations observed in the specimens examined.

Wings of workers. Due to the wrinkled and folded condition of many of the wings of all three castes, some of the groups were not discernible and the pores of many groups could not all be counted. For this reason the data for all the wings examined can not be presented. However, all five groups are invariably seen to be present when the preparations are good. These groups vary considerably in number of pores. Group 1 varies from 230 to 312, with an average of 260 for 10 wings. In three cases this group had its broader ends divided into two branches. Group 2 varies from 47 to 58, with an average of 53 for 13 wings. Group 3 varies from 156 to 192, with an average of 172 for 15 wings. Group 4 varies from 76 to 86, with 81 as an average for 15 wings. Group 5 varies from 171 to 209, with 189 as an average for 9 wings. Thus on the front wing there is an average of 485 pores and on the hind wing 270, making 755 pores on both wings of one side or 1510 on all four wings.

Legs of workers. In examining nine legs of the third pair belonging to six workers, No. 6 is always present and it is double in each case. In number of pores the smaller group varies from 8 to 10, and the larger group from 10 to 13, giving an average of 20 pores for No. 6. On the second leg this group is present on only seven of the nine legs examined; it is single in each case, but on three legs it is found on the inner instead of the outer surface. Its pores vary from 6 to 20, with 18 as an average.

This group is always present on the outer side of the first leg; it may or may not be double, and its pores vary from 18 to 20.

Group 7 of the third leg is always present, and its pores vary in number from 14 to 16. On the second leg this group may be definite or scattered, but usually has about 15 pores. This group on the first leg is always definite, with about 15 pores.

The pores of groups 8 and 9 are always considerably scattered and usually these two groups can not be separated. They are therefore not counted separately. Group 9 occurs only on the third leg and is sometimes not found even on that leg. On the third leg the number of pores of both groups varies from 7 to 21. On the second leg group 8 varies from 6 to 35 and on the first leg from 6 to 16 pores.

Groups 10 and 11 may exist as two separate and scattered groups or as one scattered group. On the third leg the pores of these two groups combined vary in number from 13 to 26, with about 16 as an average; on the second leg from 6 to 20, with 14 as an average; on the first leg from 4 to 20, with about 12 as an average.

Groups 12 to 15 are usually present on all three legs. Groups 12 and 13 are relatively constant in position and number of pores, while groups 14 and 15 are much scattered and vary considerably in number of pores. Group 12 usually consists of 15 or 16 pores, while No. 13 consists of a few less, although its pores often can not be separated from those of No. 14. The combined pores for all four groups on the third leg vary in number from 24 to 48; for the second leg, from 13 to 44, with none on the inner side of one leg, but with more pores than usual on the other side of this leg; for the first leg, 32 to 47.

Groups 16 and 17 are very similar to groups 10 and 11 except that they do not have so many pores and that on half the legs one of them is either absent or united with the other group. Their combined pores vary from 3 to 15, with about 10 as an average.

Group 18 is discernible in every case where the ventral sides of the tarsal joints directly face either cover glass, although only about a third of the tarsi happen to be turned in such a position. Usually two large pores are present on the third tar-

sal joint; sometimes only one, either on the second or third joint. These always occur in the clear spot where there are no hairs and just above the nearest place of articulation, one on either side of the median line.

For the 27 worker legs examined, the total number of pores found on the outer side of all three legs on one side varies from 153 to 211, and for the inner side from 107 to 170, giving a variation from 283 to 372 for both sides, or an average of 329.

Stings of workers. The pores on both sides of the stings of 15 workers were counted, and in location and number they vary greatly in group 19. There are usually only a few at the tip of the shaft, while most of them lie at the distal end of the bulb (fig. 14, A, c) and only occasionally are there any pores at the point marked *b*. Group 20 is constant and definite, with a pore at the base of each of the 10 or 11 barbs. Group 21 is also constant and definite, but may vary in number of pores from 6 to 12, with 9 as an average. Sometimes there are 2 or 3 scattered pores on the oblong plate (fig. 14, A, *Ob*). For the 15 stings examined, the number of pores on both sides varies from 84 to 128, with an average of 100.

On the four wings, six legs, and the sting of an average worker bee there are 2268 pores.

Wings of queens. On the wings of queens group 1 varies from 200 to 249 pores, with an average of 225; group 2, from 42 to 51, with 47 as an average; group 3, from 141 to 156, with 148 as an average; group 4, from 61 to 85, with an average of 72; and group 5, from 144 to 176, with 163 as an average. To obtain these numbers five examples each of groups 1 to 3, 6 of No. 4, and 3 of No. 5 were counted. On the front wing there are 420 and on the hind wing 235 pores, making an average of 1310 pores for all four wings of a queen.

Legs of queens. On the legs of queens group 6 is double in every instance and it is always present on the outer side in all three legs. On the third legs it varies in number of pores from 15 to 20, with 18 as an average; on the second legs, from 16 to 21, with an average of 17; on the first legs, from 15 to 19, with an average of 17.

On the third and first legs group 7 occurs three out of nine times and on the second legs four out of nine times. In number of pores it varies from 6 to 16, with about 12 as an average.

Groups 8 and 9 are either totally absent or when present they consist of only a few pores which are widely scattered. When present the pores of the two groups combined vary from 2 to 19.

Groups 10 and 11 are both always present on the third legs, their number of pores varying from 9 to 19. On the second leg one of these groups is absent in four instances and their pores vary in number from 1 to 13. On the first legs one group is missing twice, their pores here varying from 5 to 13.

Groups 12 to 15 are more scattered than in the workers, and the pores are fewer in number. Only occasionally are groups 12 and 13 more or less definite, while in the workers they are quite definite. The total number of pores for all four groups varies from 2 to 41.

On the third leg either group 16 or 17 is always absent, and in one case both groups are missing. Their total number of pores varies from 0 to 9. On the second leg both groups are usually present, with their pores varying in number from 4 to 20. On the first leg one and occasionally both groups are absent, their number of pores varying from 0 to 12.

Group 18 was never discernible in the queens.

Among the nine sets of legs of queens examined the pores for the outer side vary in number from 90 to 150 and for the inner side from 55 to 117, giving a variation in number from 172 to 262, with an average of 225 pores for both sides of three legs.

Stings of queens. An examination of the pores on both sides of nine queen stings showed that their disposition is similar to that of the worker stings. The combined number of pores for both sides of these stings varies from 83 to 111, with 100 as an average.

From a count of the pores on the four wings, six legs, and sting an average queen, therefore, is found to possess 1860 of these organs.

Wings of drones. On the wings of drones group 1 varies in number from 310 to 325, with 316 as an average; group 2, from

53 to 72, with 64 as an average; group 3, from 217 to 280, with an average of 236; group 4, from 108 to 148, with an average of 115; group 5, from 240 to 290, with an average of 268. To obtain these figures the pores were counted in group No. 1 in 5 individuals, in group No. 2 in 10, in group No. 3 in 11, in group No. 4 in 14 and in group No. 5 in 6. For the front wing there is an average number of 616 and for the hind wing 383, making an average total of 1998 pores for all four wings of a drone.

Legs of drones. On the legs of drones group 6 is constant and is always double on the third legs; its pores vary in number from 17 to 25, with 20 as an average. On the second legs group 6 is absent three times out of nine, and once it occurs on the inner instead of the outer side. When present here it is always single and its pores vary in number from 9 to 16. On the first legs this group is missing only once. It is always double and its pores vary in number from 17 to 20.

Group 7 is missing once on the third legs and twice on the second and first legs. For the third legs its pores vary from 11 to 19, for the second legs from 16 to 23, and for the first legs from 17 to 21.

Groups 8 and 9 are much scattered and are difficult to separate. Group 8 is almost always and 9 is only occasionally present. Their combined pores vary from 0 to 27 for any leg.

On the third legs groups 10 and 11 almost always exist as one scattered group, with pores varying in number from 13 to 22. On the second legs both groups are generally present, their pores varying in number from 9 to 24. Both groups are always found on the first legs. Here their pores vary in number from 10 to 18.

Groups 12 to 15 are invariably present and are considerably scattered, so that it is impossible sometimes to separate them. Group 12 is more or less definite, while group 13 always merges with group 14. The total number of pores of all four groups for the third legs varies from 11 to 49; for the second legs, from 5 to 46; for the first legs, from 12 to 45.

Groups 16 and 17 are similar to groups 10 and 11 of the outer side and in number of pores they have about the same variation.

Group 18 was discernible on all the tarsi. At least two and sometimes three large pores were present on the third tarsal joints.

As the drone has no sting, the fleshy external part of the penis of several drones was carefully examined. This portion of the penis is always covered with hairs but no pores were found.

For the nine sets of legs, on the outer side there is a variation from 154 to 201 and for the inner side from 106 to 148, making a total variation from 265 to 349 for both sides, with an average of 303 pores for both sides of three legs. On all six legs and all four wings the drone possesses an average of 2604 pores.

The thorax, abdomen, head, head appendages—in fact all parts of the chitinous skeleton—of several workers, queens, and drones were examined. Of these appendages only the mouth parts contain porelike organs similar in external appearance to these pores. They are not included in this paper, although they may be olfactory pores.

Summary

For workers, queens, and drones these pores are found on both sides of the bases of all four wings, scattered rather profusely on both sides of the trochanter of all the legs, on the outer side of the proximal end of each femur, on both sides of the tibia at the proximal end, on the second and third tarsal joints, and considerably scattered on the shaft and lancets of the sting. The five groups of pores on the wings are always present, those on the femur, tibia, and sting are generally present, while those on the trochanter and tarsal joints may be partially or almost wholly absent. Generally speaking, all of the 21 groups are nearly always present, are constant in position, but vary considerably in the number of pores.

To compare the total number of pores on one side of an individual with the total number on the other side of the same specimen, or with either side of different individuals, it has been ascertained that the variation in total number of pores is considerable.

This variation is nearly the same for workers and drones, but is much greater for the queens.

As a rule, any group contains at least a few more pores on the drone than on the worker or the queen, and the trochanters of the drone possess many more pores than do those of the queen and several more than do those of the worker. Nevertheless, since a drone has no sting and consequently lacks the organs belonging to it, the total number of pores is correspondingly lessened. The individual variations for the total number of pores of all three castes are slight.

The number of pores for each group on the wings for the three castes varies, while on the legs of all three castes the total number of pores is relatively constant. In all three the size of the groups and even the dimensions of the pores are relatively the same. Hence, since drones have the most pores and the queens the least, the pores in the former are the most densely grouped and those of the queens the least.

The following table shows the average number of olfactory pores on all four wings, on all six legs, and on the sting of the three castes of the honey bee and also the average total number of pores for each class.

TABLE 1

Average number of olfactory pores on the wings, legs and sting of the honey bee

CLASS	NUMBER OF PORES ON ALL FOUR WINGS	NUMBER OF PORES ON ALL SIX LEGS	NUMBER OF PORES ON STING	TOTAL NUMBER OF PORES
Drone.....	1998	606		2604
Worker.....	1510	658	100	2268
Queen.....	1310	450	100	1860

Since the queen possesses an average of 1860 pores, the worker an average of 2268, and the drone an average of 2604, it seems probable that whatever function they perform this is best developed in the drone and least in the queen, in proportion to the average number of pores.

Various kinds of sense organs exist on the antennae and mouth parts of the bee. Neither the external appearance nor the internal structure of any of the antennal organs has the slightest

resemblance to these olfactory pores. Janet ('11 a) speaks of a chordotonal organ at the base of the antenna in the bee, but it has no resemblance to the olfactory pores. Hicks ('60) also found some pores on the proboscis and palpi of the honey bee and of other insects. Breithaupt ('86) also describes some pores found on the base of the bee's proboscis. The drawings of the last two authors are not clear, so far as the anatomy of the pores is concerned. These same pores are found not only at the base of the proboscis but elsewhere on this appendage and also on the labial palpi and maxillae. Their superficial appearance is similar to the olfactory pores. Briant ('84) describes sense hairs on the proboscis and maxillae of the honey bee; these hairs, however, have nothing in common with the olfactory pores. Packard ('89) and Nagel ('97) find hairlike sense organs in the buccal cavity of various insects, but their structure does not resemble that of the olfactory pores. In all probability none of these organs on the head appendages is homologous in structure and function to the olfactory pores, except those on the mouth parts, but a more critical study of them would be required before anything definite could be stated concerning them. While it would not be surprising to find the olfactory pores in places other than those already named, nevertheless it is believed that nearly all, if not all, have been found.

STRUCTURE

External structure

When examined under a low-power lens the olfactory pores may be easily mistaken for hair sockets from which the hairs have been removed. When more carefully observed, even under a low-power lens, a striking difference in external form is seen. The five groups on the wings are quickly detected, but those on the legs and sting are not so readily found. If the preparation is dark, the pores appear as small bright spots, when a strong transmitted light is used. Looking at these areas under a high-power lens each one appears to have a dark boundary, or pore wall (fig. 5, *PorW*). Near the center of this boundary is a trans-

parent spot, which may be round, oblong, or slitlike. On the wings these transparent spots are always round or oblong (fig. 5, *PorAp*) and never slitlike as on the legs (fig. 12, A, *PorAp*). By slowly focusing down on this transparent area it appears to diminish in size and at the lowest level is a transparent spot, which is perfectly round on the wings and legs. This spot is an opening in the chitin, the pore aperture (*PorAp*). If the specimen examined is light, the dark pigmented pore border is more noticeable. On the wings this border is narrow (fig. 5, *PorB*) while on the legs, where the pores are not so close together, it is wider and when the pores are near each other the borders (fig. 12, A, *PorB*) merge and appear more conspicuous.

The chitin between the pores on the legs is marked, as elsewhere, with light colored lines (fig. 12, A, *ChM*). These markings on the chitin are never present between the pores on the wings but occur everywhere outside the groups. These markings on the chitin have been omitted in all the drawings except figure 12, A.

In comparing a hair socket with an olfactory pore the following differences are noticed; the largest pore is never as large as the largest socket, and the boundary of the hair socket (fig. 12, A, *PorWHR*) is always much darker and heavier, its aperture is never slitlike, and even if round or oblong it is ragged, showing where the hair has torn the edges when pulled out. As a rule the border of the hair socket (*PorBHR*) and even the chitin inside the boundary are much darker than are comparable parts of the olfactory pore. The borders of the hair sockets are usually much narrower than are those of the pores. All these differences are fairly constant, but the sockets of the smallest of the spinelike hairs (fig. 15, *SpHR₃*) on the legs are almost identical in appearance with the smallest pores and they are distinguished only by the ragged condition of the sockets. In figure 15, on the trochanter, the various kinds of hairs and the pores which belong to groups 7, 8, and 9 are shown. The pores are shown with slitlike openings while the hair sockets have irregular rings round the sockets. If these pores were drawn with dark borders, as they really appear when seen through the microscope and as the

hair sockets are represented in this drawing, the difficulty of observation would be appreciated.

From the above it is seen that the superficial appearance of an olfactory pore is that of a small round or oblong bright area that has a dark boundary and a round, oblong, or slitlike trans-

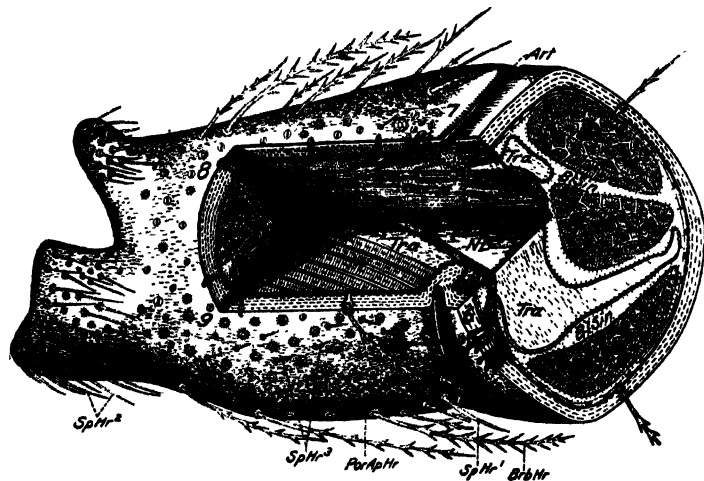


Fig. 15 Diagram of a longitudinal transverse view of the trochanter and a small portion of the femur from the third leg of a worker bee, showing the location of the olfactory pores on the outer surface as indicated by the numbers 6 to 9; also the hairs and the anatomy of the leg are shown.

parent opening. Outside the boundary there is a comparatively dark border, and inside the border the chitin is very light in color. The aperture, according to its shape, may be likened to a round, slightly flattened, or considerably flattened funnel.

Internal structure

A typical olfactory pore of the honey bee is an inverted flask-like structure in which the bottom of the flask forms the external covering or chitinous layer (fig. 16, A, *ChL*) of the pore. This layer contains the pore aperture (*PorAp*). The neck (*NkFl*) of the flask is wide and the mouth (*MF*) is flaring. About two-thirds of the space at the bottom of the flask is occupied by a chitinous cone (*Con*). The cone is not separated from the walls

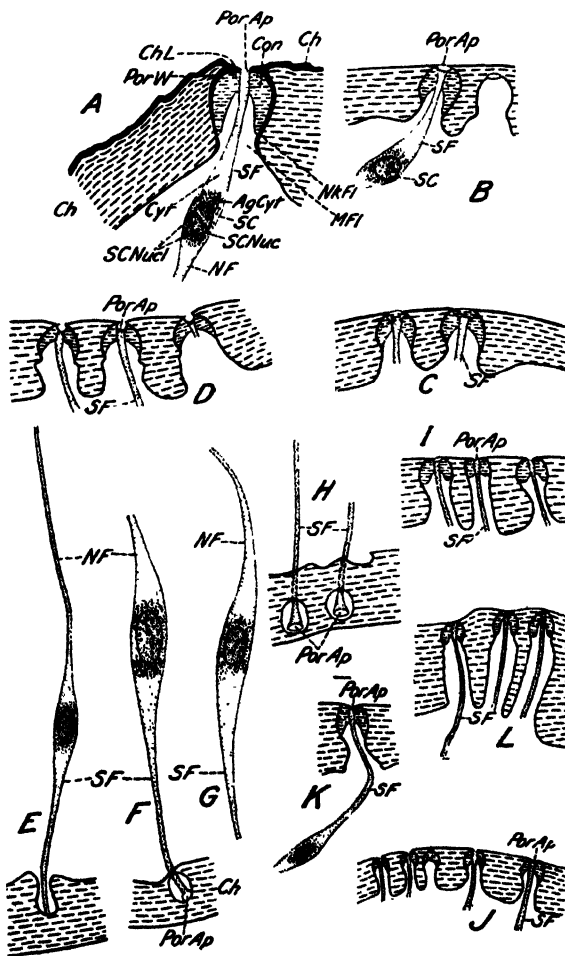


Fig. 16 Internal anatomy of olfactory pores of a honey bee, $\times 700$. A, a typical pore with its sense cell (SC) as actually seen in a section through the tibia. The longitudinal view of the pore aperture (PorAp) is shown. The edge of the chitin (Ch) and the walls of the flask (PorW) are drawn in solid black, since this part of the chitin stains more deeply. In other places in this and other drawings the chitin is represented by broken lines while the cones (Con) are shaded with fine, broken lines. The cytoplasm (Cyt) of the sense cells is represented by dots. ChL, chitinous layer; MFL, mouth of flask; NkFl, neck of flask; SF, sense fiber; NF, nerve fiber; SCNuc, sense cell nucleus; SCNucl, sense cell nucleoli; AGCyt, deeply staining cytoplasm. B, two pores and one sense cell from a cross section of the femur of the third leg; this section is one of the five included in figure 17

(*PorW*) of the surrounding flask, but it is evidently somewhat different in composition from the surrounding chitin (*Ch*), since it generally stains less deeply with iron hematoxylin and eosin or with safranin and gentian violet. The apex of the cone is hollow and extends to the neck of the flask. The sense cell (*SC*) lies beneath the mouth of the flask. It is bipolar, long, and slender, and comparatively large. The peripheral pole or sense fiber (*SF*) of this cell runs into the hollow of the cone, pierces the bottom of the cone, and enters the lowest portion of the transparent pore aperture. The inner pole or nerve fiber (*NF*) of the sense cell joins a nerve cord. The nucleus (*SCNuc*), with its several dark staining bodies or nucleoli (*ScNucl*), is always conspicuous. While the cytoplasm (*Cyt*) in the poles is almost invisible, it is easily seen at either end of the nucleus, for here it stains rather deeply (*AgCyt*).

The flasks may be short and thick or long and slender, depending on the thickness of the chitin. In the legs they are generally short and thick (figs. 16, A-C, and 17) and have a short neck. Occasionally the basal portion of the flask is short, making a long neck whose walls are slightly evaginated (fig. 16, D). In the wings and sting the basal portion of the flask is short, but the neck is usually long and narrow (fig. 16, I-K; figs. 19-21, 23), and its walls rarely have a small evagination. In the femur and tibia the pores never pierce the thickest portion of the chitin, but in the trochanter, wings, and sting this may or may not be true.

The cone is always present. Its size and shape depend on the size and shape of the basal portion of the flask. With safranin

and passes through group 6; the circles at the top represent the slits cut crosswise. C, two more pores from group 6, but here the slits are shown in longitudinal section. D, three pores from trochanter of third leg. E, F, two sense cells from two different sections of group 2 on the front wing; the chitin is cut obliquely. G, a sense cell from group 5 on the hind wing. H, two sense fibers entering pore apertures belonging to group 2 from front wing; the chitin is cut obliquely. I, three pores from group 3 from front wing entering pore apertures. J, five pores from group 4 on the hind wing, showing how sense fibers enter pore apertures. K, a typical pore and a sense cell from group 5 on hind wing. L, three pores and their sense fibers from a cross section of the tegula. Drawn with the aid of a camera lucida.

it invariably stains less deeply than the surrounding chitin, but with iron hematoxylin there is scarcely any difference in coloration.

Since the pore apertures are so small only occasionally does the microtome knife pass through the lowest part of the aperture. On account of this it is quite difficult to find a sense fiber that runs into the aperture. Nevertheless, when many sections are critically studied, it is possible to see several such connections (fig. 16, A, I-K). Sometimes the slit is shallow (fig. 16, A) and at other times it is deep (fig. 16, C').

As a rule the sense cells are conspicuous. In most cases their sense fibers are broken so that it is generally difficult to trace a sense fiber from the sense cell into the pore, although in these sections almost every pore contains at least a small portion of the sense fiber (*SF*, fig. 16, C', D, H-J). As compared with the hypodermal cells (figs. 17 and 19) the sense cells are large and in most cases lie parallel or oblique to the inner side of the hypodermis. Since most of them lie rather distant from the pores, their sense fibers (fig. 19, *SF*) are long. Their nerve fibers (fig. 19, *NF*) also are long. The size of these cells is not necessarily determined by the size of the pores, because in the legs the pores (fig. 16, A and D) are larger than those (fig. 16, I and J) in the wings where the largest sense cells (fig. 16, E-G) are found.

Many sections, each 6 microns thick, are required to contain all the pores found in group 1 on the front wing. These sections (fig. 19) show that groups 1 and 2 do not lie in the same plane as they appear to lie when observed externally. On the contrary, they lie in two different planes that are almost perpendicular to each other. In other words, the subcosta (fig. 3, *SC*) near its distal end bends at right angles and the radius (fig. 19, *R*) is connected with the subcosta only on the dorsal side. The great size of group 1 is also shown by the fact that most of its sense cells (fig. 19, *SC*) are cut transversely while only a few are seen in a longitudinal view. Five sections, each 10 microns thick, include all the sense cells belonging to group 6 on the femur of the third leg. Thus this group is about 50 microns wide.

In these five sections of a 19-day-old pupa 22 pores and 28 large sense cells (fig. 17, *SC*), besides several smaller nuclei (*SCNuc'*) were counted.

In the leg of a 19-day-old pupa the hypodermis (fig. 17, *Hyp*) is never any thicker beneath the pores than elsewhere in the*

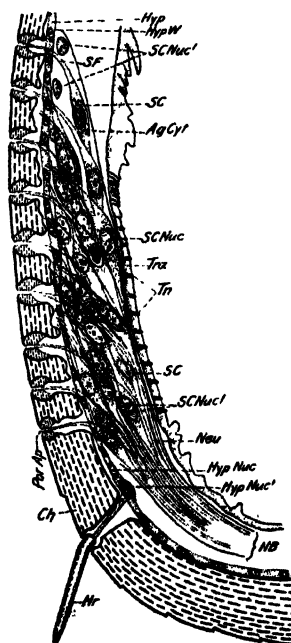


Fig. 17 Combination drawing reconstructed from five consecutive cross sections through femur of third leg of a worker pupa 19 days old. The pores and chitin are drawn diagrammatically and the hair (*Hr*) with its mother cell (*HypNuc'*) were taken from a similar section of a 17-day-old pupa. The other parts were drawn with the aid of a camera lucida. This shows the internal anatomy of group 6. *Hyp*, hypodermis; *HypNuc*, hypodermal nucleus; *HypW*, inner hypodermal wall or remains of basal membrane; *NB*, nerve branch; *Neu*, neurilemma; *Tn*, tenidia of trachea; *Tra*, trachea. $\times 465$.

leg, and even at this age it is thin and sometimes nearly wanting. Its small nuclei (fig. 17, *HypNuc*) are widely separated and the hypodermal cell walls (fig. 19, *HypCW*) are never distinct, but appear as an indefinite mass of minute lines, which are represented in most of the drawings by dots. In the legs of more

advanced pupae the hypodermis is still thinner and seems to be entirely absent at places. In the legs of younger pupae it is much thicker. Beneath the pores in the wings the hypodermis is much thicker than elsewhere (fig. 19, *Hyp*). In none of these sections of bees older than the 16-day-old pupae was it possible to distinguish a true basal membrane of the hypodermis, although it is very probable that the inner hypodermal wall (fig. 17, *HypW*) is the remains of this membrane. In younger stages of the pupae this membrane is present.

In the 16-day-old worker pupae neither pores nor sense cells are found. At this age many large and deeply staining nuclei occur in the hypodermis where the sense cells later appear. All stages in the formation of hairs may be found. The muscles are not yet completely differentiated and appear as more or less indefinite masses.

In all the sections of legs from the 17-day-old worker pupae, pores are always found, and sense cells are usually present. The chitin and most of the hairs are now fully developed. The hypodermis is much thinner than in the 16-day-old pupae, and in fact most of the various structures are fully differentiated. Thus it is evident that between the sixteenth and seventeenth days there is a rapid change in the various structures and that at this time these sense organs make their appearance. Very often in this and later stages the inner hypodermal wall (fig. 19, *HypCW*) is absent beneath the pores. In such cases it is sometimes impossible to distinguish hypodermal nuclei from sense cell nuclei. The sense cells, therefore, are derived from the hypodermis and are nothing less than modified hypodermal cells that have assumed a different function. In regard to the formation of the pores and cones nothing can be stated because this phase of the subject was not studied.

In sections from the more advanced pupae and in those from the adult bees, pores and sense cells are always present. Very often in sections of legs from pupae 17 days old a large, dark nucleus (fig. 19, *HypNuc*) is present beneath a hair. At first it is easily mistaken for a sense cell belonging to the hair, but a careful investigation always shows that it is the hair mother cell

that has formed the hair. No hairs with sense cells were found in any of these sections.

In the front wing of a 19-day-old pupa the sense cells (fig. 19, *SC*) belonging to groups 1 and 2 occupy only about one-half of the internal space. This leaves a large cavity, the blood sinus (*BlSin*) for the blood, blood corpuscles (*BlCor*), and small tracheal branch (*Tra*). Here as elsewhere the sense cells are surrounded by a membrane, the neurilemma (*Neu*), which appears as a single line. Often its nuclei (*NeuNuc*) are discernible. In the front wing of the adult bee the sense cells (fig. 20) belonging to groups 1 and 2 occupy nearly all of the internal space and leave only a small cavity for the tracheal branch and blood. In the hind wing of both the pupa and adult the space is relatively larger. This indicates not that the sense cells of the adult have increased in number, but in size. Since all the chitinous structures are completely formed by the time the bee emerges from its cell, the same number of pores would be expected in bees just emerged as are found in adult ones. This was ascertained to be the case, as was stated in discussing the disposition of the pores in the worker.

The nerve fibers of the sense cells run immediately into a nerve branch (figs. 15 and 18, *NB*) where as individual fibers they can not be traced farther. However, a longitudinal view of a nerve shows that it is composed of many parallel fibers. In the leg a branch of the nerve proceeds to each group of sense cells, but single fibers (fig. 18, *NF*) run to the isolated sense cells. No two of these branches are given off at the same time. A double-walled membrane, the neurilemma (fig. 18, *Neu*) surrounds the nerve (*N*) and its branches. The walls of the neurilemma contain many small deeply-staining nuclei (*NeuNuc*). Inside these walls among the nerve fibers there are many larger but less deeply-staining nuclei (figs. 18, 20, and 21). A cross section of the nerve shows a network of minute fibers, among which several small dark nuclei (fig. 18, *NeurNuc*) stand out conspicuously. These nuclei in both the nerve and its branches are probably neuroglia nuclei.

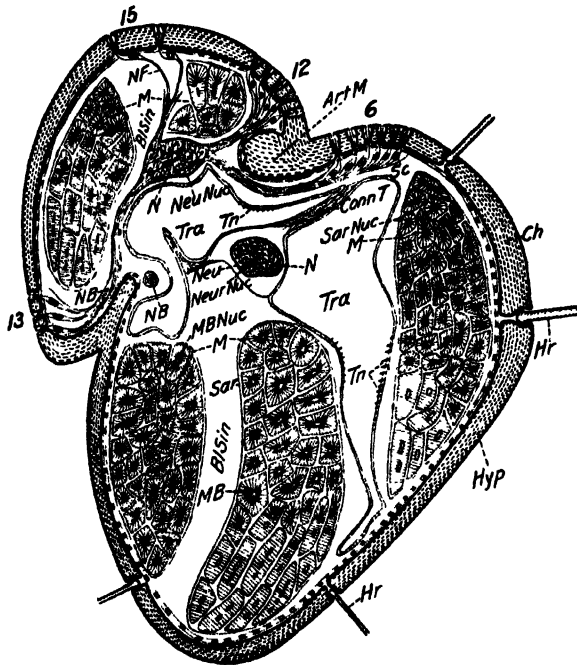


Fig. 18 Semidiagrammatic drawing reconstructed from fourteen consecutive sections through the third leg of a 19-day-old worker pupa. The trochanter and femur were bent at their articulation (*ArtM*) so that they formed an angle of 70 degrees; the sections pass obliquely through this articulation. The smaller portion at the top is the trochanter, only the inner side of which is represented. On this surface are shown groups 12, 13 and 15. The lower portion shows both the inner and outer sides of the femur, group 6 being located on the outer side. The internal parts show the innervation of the olfactory pores and the anatomy of the leg at this place. Cross sections of muscle bundles (*MB*) are shown with lines radiating from the muscle bundle nuclei (*MBNuc*), while longitudinal sections are represented by transverse lines. On account of the bend in the leg the nerve (*N*) is cut transversely in two places.

In the wings of adult bees the nerve is easily traced from the sense cells to the thorax. In one of the sections the innervation is very plain. Here the sense fibers run from the pores to the sense cells from which a nerve (fig. 22, *N*) proceeds all the way to the thorax.

The wing (fig. 22) arises at the bottom of a comparatively large niche in the side of the thorax, then for a short distance it

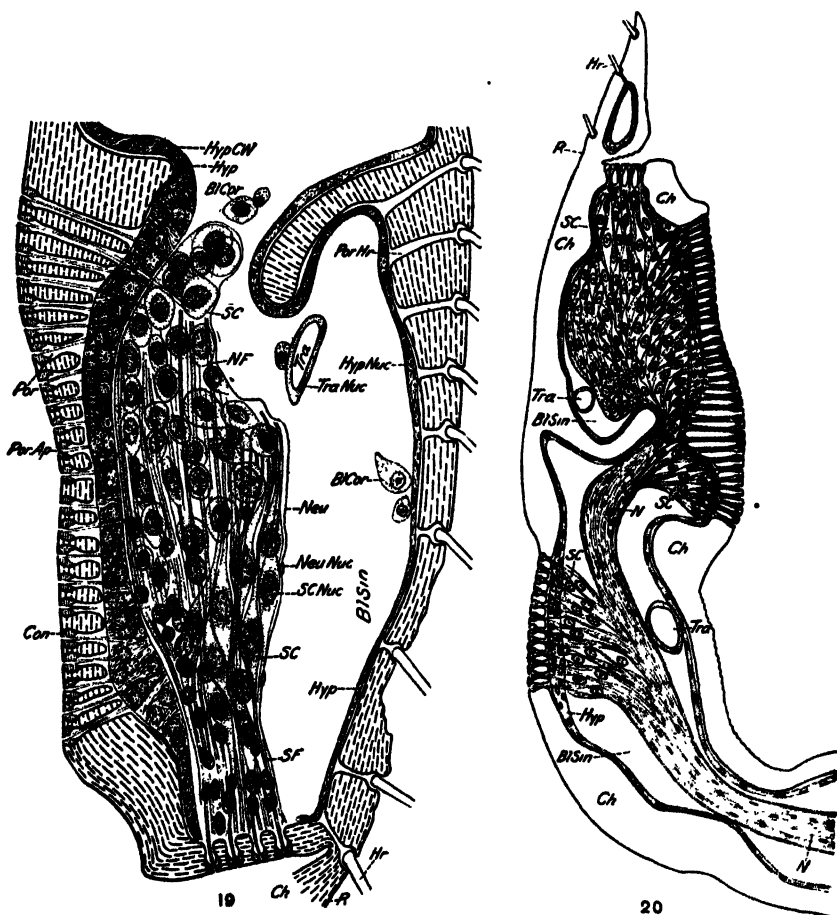


Fig. 19 Cross section (6 microns thick) through the base of a front wing of a 19-day-old worker pupa, showing the olfactory pores and sense cells belonging to groups 1 and 2. The longer row of pores belongs to group 1. All parts were drawn with aid of a camera lucida, except the pores (*Por*), hairs (*Hr*) and hypodermal cell walls (*HypCW*). The correct number of pores is represented; the hairs are drawn as they appear in another similar section, and the hypodermal cell walls are represented only by fine lines. $\times 465$.

Fig. 20 Semidiagrammatic drawing from two sections showing the innervation of the three groups of pores on the front wing of an adult worker bee. The longest row of pores is from group 1 and the shortest row is from group 2. The portion showing groups 1 and 2 is from the left front wing and the portion showing group 3 is from the right front wing of the same imago worker. $\times 185$.

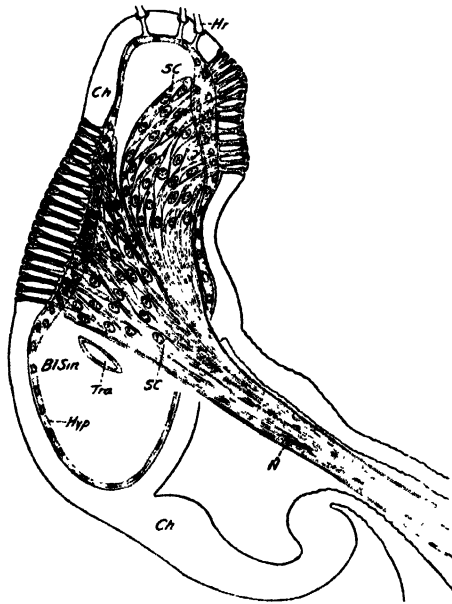


Fig. 21 Semidiagrammatic drawing from two consecutive sections of the hind wing of an adult worker bee, showing the innervation of groups 4 and 5. The longer row of pores is from group 5. The two groups of sense cells (*SC*) are drawn only approximately in their proper places, for they actually lie slightly one above the other; the trachea (*Tra*) is also shown slightly out of its normal position. $\times 185$.

runs parallel with the side of the thorax to the top of the niche, where it turns at tight angles away from the body. Since the muscles that move the wing are attached only to the sides of the thorax at the lowest part of the wing, muscular tissue is never seen in sections of the wings. The large tracheal branch (fig. 22, *Tra*) in the wing is also easily traced from the thorax to the sense cells, although a short distance from the thorax it is cut obliquely into small pieces. Its small branches near the sense cell groups are usually cut transversely but sometimes obliquely. These branches do not follow a straight path and do not extend farther into the wing than the sense cells. Tracheal branches do not persist in any of the veins or even between the articular

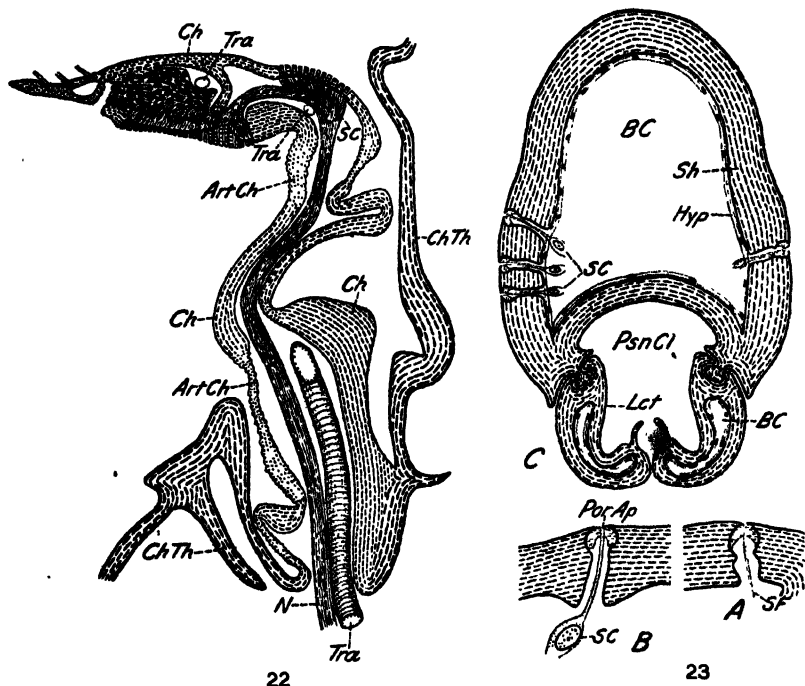


Fig. 22 Semidiagrammatic drawing from a single section of the same imago worker as in figures 20 and 21. This represents the complete innervation of the base of the front wing. The hard chitin (*Ch*) of the wing is shaded with small broken lines, the articular chitin (*ArtCh*) with dots, and the hard chitin of the thorax (*ChTh*) with heavy broken lines. $\times 78$.

Fig. 23 Olfactory pores and sense cells (*SC*) seen in cross sections of the sting of a 19-day-old worker pupa. A, pore and its sense fiber (*SF*) from shaft at *c*, $\times 700$. B, pore and sense cell from shaft midway between *c* and *e* (fig. 14 A), $\times 700$. C, cross section through the shaft (*Sh*) and lancets (*Lct*) of a sting, showing the olfactory pores and their sense cells, $\times 350$. In C, the pores, sense cells, and hypodermis are drawn diagrammatically. The remaining parts of C and all of A and B were drawn with aid of a camera lucida.

chitinous layers, whether nearly or completely developed, except where they are associated with the sense cells.

The large nerve of the leg is also easily traced to the thorax. By using several of the sections from the thorax of an adult worker these nerves were followed to large ganglia in the thorax. According to Snodgrass ('10) the prothoracic ganglion innervates the prothorax and the first pair of legs. The second thoracic

ganglion which is a combination of the mesothoracic, metathoracic, and the first two abdominal ganglia, innervates the second and third pairs of legs, the bases of both pairs of wings, the mesothorax, metathorax, the propodeum, and the first abdominal segment.

The groups of sense cells are so located that they are constantly bathed in the blood. The large trachea (figs. 15 and 18, *Tra*) branches profusely, and one of its branches is always near a group of sense cells. There seems to be little connective tissue (fig. 18, *ConnT*) in the leg of a bee. It appears as fine lines and is found between the tracheal branches and nerves. The large muscles (figs. 15 and 18, *M*) are so located that they do not interfere with the sense cells while contracting and relaxing. Hence the sense cells are so located that they are constantly nourished by the blood that surrounds them and they receive their supply of air from the neighboring tracheal branches. Also, the muscles are distant enough not to affect them mechanically.

At various times while examining the superficial structure of the pores in group 3 on the front wing, peculiar structures which were first thought to be hair sockets were noticed irregularly scattered on the tegulae (figs. 2 and 6, *Tg*). Cross sections through these apparent hair sockets (fig. 16, *L*) show that they have the same structure as the olfactory pores. They were observed in only a few sections and their sense cells were never seen. On account of the small size of these structures and since their apertures are never distinct, they were at first not considered as olfactory pores, but since they are identical in structure with the olfactory pores, even if their sense cells were not seen, it is evident that these structures are the external ends of olfactory pores.

Cross sections of stings were made in the same manner as those of the legs and wings, although the slides fail to reveal any sections passing through the extreme end of the lancets.

Since the superficial appearance of the pores in groups 20 and 21 on the lancet (fig. 14, *A*, *F*, and *G*) is identical with that of the pores on the shaft, it may be assumed that the internal anatomy of the pores on the lancet is identical with that of the pores on the

shaft. From the drawings (figs. 16 and 23) it is clear that the structure of the pores on the sting is identical with that of the pores on the legs and wings, and that the pores of the sting open internally into the body cavity (*BC*) and not into the poison canal of the sting (*PsnCl*).

In regard to the pores of the sting Snodgrass ('10) says:

The reader . . . will see often repeated the statement that the poison leaves the sting both by a ventral opening between the lancets near their tips and by several lateral pores near the ends of the lancets opening from the poison canal upon the bases of the barbs. The writer, however, has never been able to observe the exit of the poison from any such lateral pores, while, on the other hand, it is very easy to watch it exude from between the lancets on the ventral side of the sting near the tip.

An examination of the end of each lancet does reveal a number of oblique pores which have been figured by other writers, and they certainly open on the bases of the barbs as described, but their inner ends apparently communicate with the body cavity (*bc*) of the lancet instead of passing clear through the lancet and opening into the poison canal. Furthermore, a paired series of exactly similar pores extends the entire length of the shaft of the sheath, opening on its dorsal surface from the body cavity. No one could possibly claim that the poison emerges also through these pores, which, very curiously, do not appear to have been described before, although they are even more conspicuous as well as more numerous than those of the lancets. The writer has not been successful in preparing histological sections of the sting which show these pores, but they probably constitute the ducts of some kind of subcuticular glands.

From the foregoing quotation it is clear that Snodgrass considers these pores as communicating with the body cavity of the shaft and lancet and not of the poison canal. But instead of being some kind of a subcuticular gland, they are connected with sense cells and are thus sense organs.

Summary

To sum up, then, the olfactory pores consist of inverted flasks in the chitin and spindlelike sense cells lying beneath the mouths of the flasks. The bottom of the flask forms an external covering and about two-thirds of the space at the bottom of the flask is occupied by a hollow chitinous cone which is not separated from the surrounding chitin, but only stains less deeply.

The sense fiber of the sense cell pierces the bottom of this cone and enters the round, oblong, or slitlike external chitinous pore aperture. The morphology of the pores and the sense cells in the wings, legs, and stings is identical, with the exception of variations in size and shape. The complete innervation for those in the wings and legs is also identical. The interior of the base of the wings is literally a solid mass of sense cells while in the legs and stings these cells occupy comparatively little space. In the worker bee the pores and sense cells are first noticed in the 17-day-old pupa, and the sense cells do not reach their full size until the bee arrives at the adult stage, although all the pores seem to be present in the 19-day-old pupa.

Judging from the structure of these organs it is observed that the cytoplasm in the end of the sense fiber just beneath the pore aperture is constantly in contact with the external air. In speculating as to the function of such an apparatus, all mechanical senses, such as sight, touch, and hearing, must be excluded, and these organs may be presumed to react to some kind of a chemical stimulus, as humidity, temperature, taste, or smell. Without hesitation taste may be excluded, for the food of a bee never comes in contact with the parts containing these pores.

PHYSIOLOGY OF OLFACTORY PORES

To ascertain whether the pores which have been studied are actually the organs in the bee that receive odor stimuli, the wings, legs, and stings of many workers were operated upon. The behavior of these individuals was carefully studied and they were tested with various odors as described on page 280.

Wings cut off

All four wings of 50 middle-aged workers were cut off close to the thorax with small dissecting scissors. An examination of these wings showed that only occasionally did the scissors pass through a group of these pores. These bees were entirely normal, judging by their behavior and longevity. They lived from 18 hours to 16 days, with 9 days and 23 hours as an average.

When tested with odors they responded normally, but slightly more slowly. The average reaction time to oil of peppermint was 2.4 seconds, to oil of thyme 3.3 seconds, to oil of wintergreen 3.5 seconds, and the average 3 seconds. This is 0.4 second slower than the general average of unmutilated bees with the same odors. The workers tested were 17.

Wings pulled off

All four wings of 150 middle-aged workers were pulled off. This is accomplished by holding the bee firmly on a table and by giving each wing a quick jerk, severing it at its articulation. A microscopical examination of the detached wings showed that they possessed all the groups of pores. Judging by their behavior and longevity these wingless workers were otherwise normal in every way. When first introduced into observation cases, they immediately busied themselves in cleaning each other, during which operation they often licked up the drop of yellowish blood that exuded where the wing was detached. They also ate freely, fed each other, never fought, and, in fact, behaved as normally as unmutilated bees in such surroundings. They lived from 3 hours to 17 days, with 9 days and 20 hours as an average.

Strange bees which were put among them were at once recognized as strangers. The wingless ones ran after the strangers, bit their legs and pulled their wings, but only occasionally did a wingless bee seize a stranger, and not once did one attempt to sting a strange worker. This treatment lasted only a few minutes, for in a short time the wingless bees fed, cleaned, and even stroked the strangers with their antennae. The behavior toward strange bees was entirely different than that shown by those with their wings cut off just beyond these pores. Pulling off the wings later proved to be a good precaution before mixing strange bees. It is slow but always effective, because they never kill one another even though they may be slightly hostile at first.

These wingless bees were tested with odors in the usual manner. Their responses, when they reacted, were similar to those

of uninjured bees, but always much slower. They often failed to respond at all, although they were otherwise as active as unmutilated bees. A failure to respond within 60 seconds after an odor was held under them was regarded as entirely negative for that odor. The most general responses were to move away slowly and to vibrate and stroke the antennae. The average reaction time to oil of peppermint was 18.8 seconds, to oil of thyme 20.6 seconds, to oil of wintergreen 27.3 seconds, to honey and comb 28.8 seconds, to pollen 38.5 seconds, and to leaves and stems of pennyroyal 28.8 seconds. The average was 27.1 seconds. Twenty-eight workers were tested. For unmutilated bees with the same odors the general average reaction time was 3.4 seconds; therefore by removing the pores on all four wings the average reaction time is increased eight times.

Bases of wings glued

For fear that the bees with their wings pulled off were abnormal in some unknown way, the bases of all four wings of many workers were glued by applying a small amount of liquid glue to each side of the wing near the articulation. When the glue was thoroughly dry these workers were put into observation cases. The glue caused the wings to be stiff so that they could not be vibrated, but judging from their behavior and longevity the bees were normal in all other respects. They lived as long as unmutilated bees, and when strange workers were put among them they were always accepted without much hostility. The responses were the same as for the wingless bees. The average reaction time to oil of peppermint was 12.5 seconds, to oil of thyme 15.6 seconds, to oil of wintergreen 27.5 seconds, to honey and comb 38.8 seconds, to pollen 36.8 seconds, and to leaves and stems of pennyroyal 38.8 seconds. The average was 28.2 seconds. Twenty workers were tested. Thus by covering all the pores on the wings with glue the reaction is increased eight times. That the odor of this glue does not affect the results is shown on page 296.

Pores on legs covered

The entire trochanter, the proximal end of the femur^{*}, and the tibia of all six legs of 36 workers were covered with a thin layer of vaseline and beeswax applied with a small pencil brush. The proper stiffness of the mixture was obtained by using three parts of melted yellow commercial vaseline with one part of melted beeswax. Great precaution was taken not to apply too much of this mixture, because when a bee so treated is liberated it invariably begins at once to clean itself and often rubs the mixture on the spiracles and dies quickly. After a short time these workers ceased to clean themselves, began to eat, and were quite normal in their behavior. On an average they lived as long as untreated bees and succeeded in cleaning off all the mixture by the fourth day. They were tested two or three hours after being treated and responded normally but slightly more slowly. The average reaction time to oil of peppermint was 4.2 seconds, to oil of thyme 6.2 seconds, to oil of wintergreen 5.1 seconds, to honey and comb 18.8 seconds, to pollen 9.7 seconds, to leaves and stems of pennyroyal 11 seconds. The average was 8 seconds, or two and one-half times the average for untreated bees with the same odors. Twenty workers were tested.

Since this mixture of vaseline and beeswax gives off a slight odor, there was a possibility that the odor itself caused the foregoing differences in reaction time. To ascertain this, a still larger amount of this mixture was placed on the antero-dorsal side of the abdomen, and the bees were tested with various odors. In such a position the bee can not reach it and therefore can not escape the odor of the mixture. The average reaction time to oil of peppermint was 2.5 seconds, to oil of thyme 2.6 seconds, to oil of wintergreen 3.1 seconds, and average 2.73 seconds. Eighteen workers were tested. The same odors with normal bees gave an average reaction time of 2.64 seconds. The odor of the mixture, therefore, did not affect the reactions to other odors.

Wings pulled off and pores on legs covered

For this experiment 33 of the 150 wingless bees mentioned on page 334 were used. The pores on the legs were covered with the mixture of vaseline and beeswax four days after the wings had been pulled off. When put into observation cases, these workers were quite hostile to each other, they bit and pulled one another's legs and wings for at least three hours, after which they cleaned and fed each other as usual. They were otherwise normal in all respects and lived on an average 9 days and 5 hours. When strange bees were put among them, they were quickly attacked and were soon killed. In reacting to odors their behavior was similar to that of un mutilated bees, but much slower. The reaction was at times entirely negative to all the six odors. The average reaction time to oil of peppermint was 27.3 seconds, to oil of thyme 44.1 seconds, to oil of wintergreen 39.3 seconds, to honey and comb 41.7 seconds, to pollen 44.5 seconds, and to leaves and stems of pennyroyal 42.9 seconds, and the average of all was 40 seconds. The workers tested numbered 20. By removing all the pores on the wings and by coating most of them on the legs the time of response to the six odors was increased 12 times.

The experiment just described may be criticised on the ground that the removal of the wings of bees causes an abnormal condition which is brought about by the injury of the operation, and that the reaction time is affected on account of the injury. When the behavior of bees thus mutilated and their longevity are considered it will be clear that the injury itself does not cause the slow responses. It may also be urged that any substance which is used to cover the pores comes in contact with the peripheral ends of the sense fibers and this contact might make the insect abnormal, so that even if these organs were not olfactory, the bee would not respond to odor stimuli. Such a contact is scarcely possible for two reasons: (1) Since the parts of the bee where the pores are located are covered with a dense coat of hair, the liquid glue or mixture of vaseline and beeswax probably

does not come in direct contact with the chitin around the pores; (2) as the sense fibers end in the neck of the funnel-like aperture and do not extend into the mouth of the funnel, their peripheral ends do not attain the level of the external surface of the chitin. It thus seems impossible for these thick substances to run into the mouths of the funnel-like apertures, where they must force out the air in order to come in contact with the ends of the sense fibers.

To anesthetize the ends of the sense fibers with a finely powdered anesthetic, either by blowing or by applying the powder with a brush on the parts possessing the pores, is impossible for two reasons: (1) The powder does not come in contact with the chitin on account of the dense coat of hair; (2) the diameter of the smallest particles of the powder is microscopically two or three times that of the largest pore apertures.

To anesthetize the ends of the sense fibers by using an anesthetic dissolved in water, the following procedure was employed: With a small pencil brush the pores on the legs and wings were covered with a one-fifth saturated solution of alypin, a synthetic anesthetic. The bee was immediately put into a small observation case and the latter was held in the current of an electric fan for five minutes. By this time the bee was again dry and appeared normal in most respects, but it was extremely restless and did not eat candy until several minutes afterwards. Since the bee did not become perfectly quiet until two or three hours afterwards, a reaction time in seconds could not be obtained while the anesthetization was effective. In view of the fact that the rate of walking of unmutilated bees may be increased by using odors, this anesthetized bee was tested with oil of peppermint to determine whether odors cause its rate of movement to be increased. The word excited is used to describe the action of the bee when it quickly moved from over the odor and vigorously vibrated its wings. In all, 10 workers were used, with the following results:

Two workers: During the first 10 minutes their slow rate of walking was not increased; after 15 minutes one was easily excited; after 30 minutes the other could not be excited and in-

creased its rate only slightly, and after 40 minutes it moved a little faster when tested.

One worker: During the first 3 minutes it did not increase its rate, but later was easily excited.

Five workers: During the first 5 minutes they increased their rate only slightly, but later were easily excited.

Two workers: At first, as well as later, they were easily excited.

A control was used by applying water in the same manner as the anesthetic, with another pencil brush. In all, 10 workers were used, and each one without failure was easily excited at first as well as later. It may be suggested that only a relatively small amount of any liquid is able to reach the ends of the sense fibers, for two reasons: (1) On account of the dense coat of hairs, and (2) because the liquid must replace the air in the mouths of the funnel-like apertures.

Stings extracted

. For this experiment 20 of the 100 bees with their stings extracted, as mentioned on page 278, were used. In most respects these bees appeared normal, but they lived only 30 hours on an average. They responded to odors normally. The average reaction time to oil of peppermint was 2.6 seconds, to oil of thyme 3 seconds, and to oil of wintergreen 3 seconds. The average was 2.8 seconds. This is 0.2 second more than the average for the same odors when unmutilated bees were used.

SUMMARY

Bees with their antennae mutilated in the slightest degree, either by amputation or by varnishing them, are never absolutely normal. The more joints of the antennae severed, the more abnormal the bees are. There is nothing in the results of these experiments to indicate that the antennae play any part in olfaction. The maxillae, labial palpi, proboscides, mandibles, and stings may receive odor stimuli in a limited degree but this is not certain. Neither the mouth cavity nor the epipharynx plays

any part in receiving odor stimuli. When the wings are cut off distal to the olfactory pores, the reaction time odors is not materially changed. When the wings are pulled off or the bases are glued the reaction time is increased eight times. Sister bees so treated are not hostile to each other and do not actively fight strange bees. Such facts indicate that the olfactory organs are sufficiently destroyed or prevented from functioning by these manipulations so that the bees do not readily recognize enemies. With the pores on the legs covered, the reaction time is increased two and one-half times. When the wings are pulled off and the pores on the legs are covered, the reaction time is increased twelve times. Sister bees so treated are slightly hostile to one another and quickly attack strangers. After fighting a short time they distinguish friends from enemies, cease attacking their friends, but never cease fighting their enemies until the strangers are killed. Neither the odor of glue nor of vaseline affected these results.

TABLE 2

Summary of experiments with worker bees to determine the location of the olfactory organs

EXPERIMENT	AVERAGE REACTION TIME		NUMBER OF WORKERS TESTED	AVERAGE LENGTH OF LIFE IN THE OBSERVATION CASE	
	For three odors	For six odors		days	hours
Unmutilated.....	2.64	3.4	37	9	3
Glue on thorax as control.....	2.76		19	9	3
Vaseline on abdomen as control.....	2.73		18	9	3
Flagella burnt off.....	4.0		7	0	17
Flagella glued.....	2.9		21	1	0
Maxillae and labial palpi cut off..	3.3	4.0	19	1	0
Proboscis cut off.....	2.9		22	0	7
Mandibles cut off.....	3.5	4.8	20	7	0
Flour paste in mouth.....	2.68		20	7	12
Wings cut off beyond pores.....	3.0		17	9	23
Stings extracted.....	2.86		20	1	6
Wings pulled off.....	22.2	27.1	28	9	20
Bases of wings glued.....	18.5	28.2	20	9	3
Pores on legs covered with vaseline..	5.2	8.0	20	9	3
Wings pulled off and pores on legs covered with vaseline.....	36.9	40.0	20	9	5

As the structure of these pores is suitable for receiving chemical stimuli and since all the foregoing experiments with these organs indicate an olfactory function for them, it may now be assumed that these pores constitute the olfactory organs in the honey bee, and perhaps in insects in general.

Table 2 gives the results of all experiments with worker bees in timing their responses to various odors. The "three odors" used are those from oil of peppermint, oil of thyme, and oil of wintergreen. The "six odors" used are those from oil of peppermint, oil of thyme, oil of wintergreen, honey and comb, pollen, and leaves and stems of pennyroyal.

GENERAL SUMMARY AND DISCUSSION

The behavior of bees in observation cases is very similar in many respects to that in the hives. Bees have a very acute sense of smell. This sense is most highly developed in the drones and least developed in the queen while that of the worker is scarcely inferior to that of the drone. Olfactory pores are found on the bases of all four wings, widely scattered on the trochanter and at the proximal ends of the femur and tibia of all six legs, on the second and third tarsal joints of most legs and generally distributed on the shaft and lancets of the sting. Each pore is a chitinous structure connected with a bipolar sense cell, the peripheral end of which comes into direct contact with the external air. Such sense cells are met with in all insects for Künchel and Gazagnaire ('81) assert that bipolar sense cells are common to all insects.

Bees with their antennae mutilated in the slightest degree, either by amputation or by covering the surface, are never absolutely normal. The more joints of the antennae severed, the more abnormal the bees are. The antennae probably play no part in receiving odor stimuli. The maxillae, labial palpi, mandibles, and sting either receive odor stimuli to a limited degree or the mutilation in the experiments on these parts caused a slight difference in the reaction time. Judging from their behavior and longevity, bees with the wings either pulled off or glued at the base and with the pores on the legs covered are normal, except

that their ability to smell is greatly reduced. In the honey bee these pores are olfactory organs, and perhaps the same is true for all insects because they are common to all orders of insects and possibly are homologous to the lyriform organs of spiders (McIndoo '11). As yet they have never been looked for in crustaceans, but it is very probable that this class of arthropods possesses similar organs.

The following criticisms concerning the physiological experiments performed with the antennae of various insects may be offered. Most of the previous observers have studied the behavior of the insects investigated in captivity for only a short time, while the remainder have paid no attention at all to the behavior of their un mutilated insects. They cut off either a few joints of both antennae, or these entire appendages, or varnished them with paraffin, rubber, and so forth. When a few joints are severed the sense of smell is apparently weakened. This is true for bees also. When both antennae are amputated or varnished the insects, as a rule, fail to respond to substances which normally affect the olfactory sense. They generally fail to respond to odors held near them and fail to find food in captivity, and do not return to putrid meat and dead bodies when removed from such food. Males so mutilated as a rule do not seek females and show no responses when females are placed near them. Such experiments were seriously criticised until Hauser in 1880 presented his apparently conclusive results. Many of the insects on which he experimented with the antennae amputated became sick and soon died. Most of them failed to respond when the antennae were mutilated, although *Carabus*, *Melolontha*, and *Silpha* responded slightly, while all the Hemiptera that he used responded almost as well with their antennae off as they did with them intact. Only 40 per cent of the ants from which Miss Fielde ('01) cut the antennae recovered from the effect of the shock. Not one of these observers has studied the behavior of the species under observation sufficiently to know exactly how long they live in captivity with their antennae either intact or mutilated. No one, except Miss Fielde, has kept a record of the death of the mutilated and normal insects

so accurate that one might know what percentage died from the operation. To cut off some other appendage or even the lower part of the head, as Forel ('74, '85) did, is not a fair test, because such operations seldom expose sense cells and never any nerve equal in size to that of the antenna, unless one pulls off the wings. When the wings are pulled off the large nerve is severed between the masses of sense cells and thorax, and the sense cells are not exposed to the air, as they are when antennae are removed. Even if the antennae are cut through the scape, the large masses of sense cells belonging to Johnston's organs (Child '94) are severed. When the lower part of the head or the tarsi are cut off, as Forel did, no nerves are exposed to the air except ends of small nerves. From the foregoing it is only reasonable to assume that when the antennae of any insect are injured in the least degree, the insect is no longer normal and if it fails to respond to odors placed near it, this negative response may be due to the shock of the injury.

The following criticisms based on a consideration of the morphology of the antennae may also be offered. In the honey bee the pore plates (Schenk '03) can scarcely be considered as olfactory organs, because the drone has almost eight times as many as the queen, and responds to the odors presented in slightly more than one-half the time. It is true that those of the queen are considerably larger, but even on this basis the reaction times are not comparable. The pegs may be entirely eliminated as olfactory organs, because they are absent in the drone, but are abundant in the worker and the queen. Drones, queens, and workers have about the same number of Forel's flasks and pit pegs. Schenk's view that the pegs receive odor stimuli in the queens and workers, while Forel's flasks and the pit pegs function in this way in the drones is inconsistent, because if the latter two structures function for such a purpose in the drones why should they not also in the females? Since these two structures are few in number and many times smaller than the pegs, we can not compare them physiologically. Thus it is seen that not one of these antennal organs of the honey bee offers a solution for the ratios obtained with the use of the various odors.

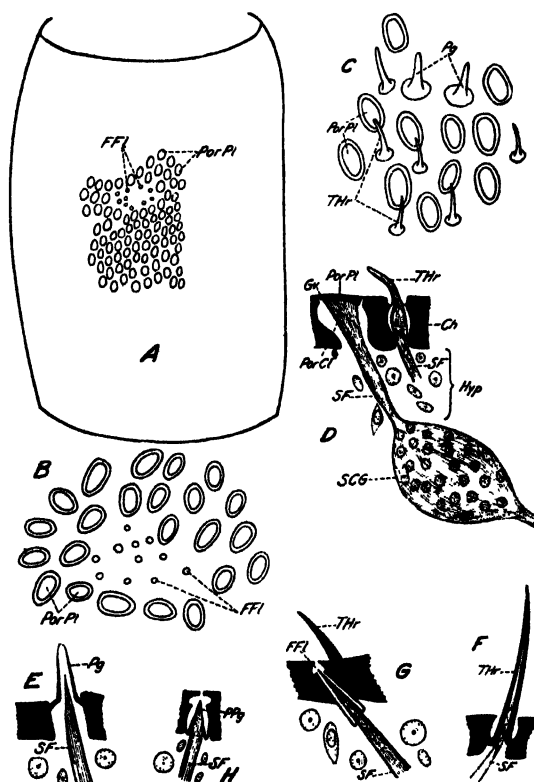


Fig. 24 Antennal organs of the honey bee copied from Schenk. A, an antennal joint of a drone, showing a few of the many pore plates (*PorPl*) and a group of Forel's flasks (*FFl*), $\times 100$; B, pore plates and Forel's flasks from a drone's antenna, $\times 400$; C, pore plates (*PorPl*), pegs (*Pg*), and tactile hairs (*THr*) from a worker's antenna, $\times 400$; D, internal anatomy of a pore plate and of a tactile hair; E, the same of a peg, F, the same of a tactile hair; G, the same of a Forel's flask; H, the internal anatomy of a pit peg. D—H, $\times 500$.

If the reaction time of each caste is compared with the total number of olfactory pores a consistent inverse ratio is obtained. A drone has 2600 pores and responds in 2.9 seconds; a worker possesses 2200 pores and responds in 3.4 seconds and a queen has 1800 pores and responds in 4.9 seconds.

Pore plates are not the olfactory apparatus in all insects, because they are entirely absent in the *Lepidoptera*. The pegs

can not be the olfactory organs in all insects, for they are absent in many male bees and almost wanting in Lepidoptera, although possibly the end rods in butterflies and moths are homologous. According to Vom Rath ('95) pegs are found not only on the antennae and mouth parts but also all over the body, and Nagel ('94) found them elsewhere than on the antennae. If the pegs are the olfactory organs and if insects with amputated antennae are normal, then why do not such insects respond positively at least slightly to odors instead of negatively, as most observers claim?

It is certain that spiders can smell, yet they have no antennae nor any organs that may be compared to the antennal organs of insects. Hence, this is another argument against the antennae being organs of smell. All insects either have antennal organs like those described for the bee, or modifications of them, yet no two authors who have studied them have agreed concerning their function. Such chaos can be replaced by facts, only when the behavior of the insects investigated is thoroughly studied and when experiments are performed in ways other than on the antennae alone. Then it will be realized that the antennae can no longer be regarded as the seat of the sense of smell in insects.

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TRANSMISSION THROUGH THE RESTING EGG OF EXPERIMENTALLY INDUCED CHARACTERS IN *ASPLANCHNA AMPHORA*¹

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The chief purpose of undertaking the experiments which form the basis of this article was to ascertain whether or not the marked modifications which may be experimentally induced in the above rotifer are in any sense hereditary beyond the parthenogenetic stages. As to what the modifications are in this remarkably variable and tri-morphic species and as to the means by which they are produced in nature as well as under experiment, we refer the reader to the previous articles in the present series, as well as to the publications of Lange, Powers, and Daday (see bibliography). We may state briefly that variation in this species is shown to be, in last resort, entirely under the control of nutrition; that in character it is deep-seated, pervasive, erratic, and sudden—bearing all the ear-marks of mutation; and that it is hereditary in a strong though variable degree throughout the parthenogenetic series.

In several respects variation in this rotifer appears like a species-making process: The different types produced occur erratically though sometimes abundantly in nature; their differences would be quite sufficient for specific distinction, and several of the forms produced closely parallel types of the genus which have been assumed to be definite species. Nevertheless it is plain that we can not regard the variation of this *Asplanchna* as a true species-forming process until it is proven that the variation in question effects, in at least some degree, the sexual gametes. However varied the parthenogenetic forms and however thoroughly it may be proven that this variation is a result of a modification of the parthenogenetic ova, which transmits itself for

¹ Studies from the Zoölogical Laboratory, The University of Nebraska, No. 110.

generation after generation, it is clear that nothing akin to a new species is formed if sexual reproduction again brings the species back to its original form.

This seems at first sight to be the case. All the fertilized or resting eggs hatch as individuals of the small saccate type, which represents in general the primitive phylogenetic form of the entire genus. The astonishing variations of the parthenogenetic series, germinal though they are, thus seem to be wiped out by sexual reproduction much as the somatic variations of the complex metazoan are wiped out by the same process. The question which we wish to answer is thus, whether this wiping out process is complete; whether the fertilized egg is so different a thing from the parthenogenetic egg; whether the entire variation of the species, and doubtless of the genus as a whole, is but a play of the environment upon parthenogenesis as such, or whether it is the result of the forces fundamentally modifying the gametic constitution of the species.

It should be pointed out that the degree of inheritance through the resting or fertilized egg must obviously be less than the usual degree of inheritance through the parthenogenetic egg, since the latter shows at least a strong tendency to maintain the species or at least the race in question in a constant physiological and morphological state. This the resting egg does not do; the first generation of young hatching from it, is always *morphologically* the same, from whatever type of the species the egg is derived. There is still, however, the possibility that the young saccate individuals derived from different eggs differ *physiologically* and in their reproductive tendencies.

Evidence has accumulated during our work on this rotifer which tends to show that a parthenogenetic series of these small animals, metazoa though they are, conducts itself very much as does a series of protozoa derived from a single conjugant. Such a series may thus be compared, with not a little truth, to the somatic development of a larger metazoan resulting from the fertilized egg. Now, just as in the metazoan, characters may crop out at late stages and after a multitude of cell generations, which are none the less determined by the inherent qualities of the ferti-

lized egg, so it is readily conceivable that in the parthenogenetic series of rotifers the tendency for certain form-changes to occur in generations more or less removed from the resting egg may none the less have been determined by the nature of that egg as such. In short, there may be hereditary. Or, to put the same thing in a different way for clearness, it is conceivable that inheritance through the resting or fertilized egg of this rotifer need not manifest itself in the visible characters of the individual which emerges from this egg; it may be hidden as tendencies only to manifest itself in later generations.

A few terms may be briefly defined as an aid to readers not familiar with our preceding studies upon *Asplanchna*. A pedigree-series is a succession of individuals derived, by parthenogenetic reproduction, from a single resting or fertilized egg. One of the early members of each generation is usually isolated to become the parent of the succeeding generation. The term metabolic rhythm has been used to indicate an internal rhythm which can be measured by the rise and fall in the number of young born, under normal conditions, to the individuals constituting the generations in the pedigree series. The words, type, form, and stage, refer to the tri-morphic conditions in which this rotifer is found. The word potential is used to indicate the different physiological levels as represented by these three different stages.

EXPERIMENTS

All the *Asplanchna* employed in the following experiments were derived from stock used in the previous year in the studies upon sex-determination. A single mass culture had been carried through the summer. Four healthy individuals were chosen from this in the fall and were tested by allowing them to multiply rapidly in a single mass culture. The stock seemed good: In a few days many had been born and even males had been produced. On October 7 a single individual was chosen to become the parent of the mass cultures from which all series and cultures used through the experiments were derived, all other material being entirely discarded.

The initial mass culture thus derived proved remarkably strong. By October 10, but three days after the isolation of the single progenitor, hundreds of rotifers were present. Males were also first seen on October 10, and the following day individuals containing resting eggs were present.

From this mass culture derived, as we have seen, from a single individual, four series, in two contrasting groups, were started as follows: First, one of the above-mentioned individuals containing resting eggs was isolated on October 11. Its eggs, dropped two or three days later, were placed in watch glasses containing fresh tap water, where they began to hatch by October 24. Two of these young were chosen to become the parents of series *H* and *I*, which, being similarly derived, constitute one group. Other sister eggs hatched and were used to found general mass cultures serving no other purpose than as subsidiary controls.

Second, another group of two series was started in a different way. The general mass culture from which the original progenitor of *H* and *I* had been chosen was fed in a special manner so as greatly to increase its numbers, to induce cannibalism, and thereby produce a number of the third or campanulate type of the species. These campanulates were active cannibals in their own culture before their isolation. After isolation they were fed upon numbers of the saccate form of their own species. Male production soon followed. As soon as this was noted a single young campanulate was isolated and fertilized by a large male produced by another campanulate mother; resting eggs were produced, and after suitable treatment began to hatch on December 2. Two of these young were used to become the direct progenitors of the two series *J* and *K*.

The relation of the above-mentioned four series should thus be clear: All are the descendents of one parthenogenetic female although many generations removed. *H* and *I* are derived from individuals which are full sisters hatched from resting eggs produced by the humped type. *J* and *K* are likewise full sisters but hatched from fertilized eggs derived from the still higher or campanulate stock. Thus the immediate progenitors of these

series, although of common descent, were of different morphological types and also of what we have called two different potentials or different physiological states artificially produced from what was at the start an apparently homogeneous parthenogenetic stock. The purpose of the experiment is to see whether the advent of sexual reproduction at the beginning of each of these series will have obliterated these different potentials, or in other words, whether the four series derived in these two different manners will be different or identical when maintained and bred under identical conditions. These four contrasting series were reared as follows: From *H* one hundred and forty-five generations; from *I*, eighty; from *J*, fifty-five; and from *K*, eighty-seven. Table 1 gives the histories of these four series.

Examination of table 1 shows the astonishing difference between the two groups—series *H* and *I* on the one hand, and series *J* and *K* on the other. Equally striking is the parallelism between the two series in each group. *H* and *I* remain alike practically throughout the entire experiment; *J* and *K* equally so. It will be seen that *H* and *I* are all but homogeneous saccate series. They are as constant as were series *D* and *D 2* recorded in the first paper of these studies. Temporary transitions to the humped type do occur, but they are rare and only at times when the metabolic rhythm was at its height. It should be distinctly stated that *H* and *I* were not weak races.

There were no signs of decadence in either series, even though *H* was carried to the one hundred and forty-fifth generation. Both series might have been prolonged had time permitted. The average number of young produced per individual approximated closely to sixteen in *H* and to fifteen in *I*.

TABLE I
History of pedigree Series H

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOW- ING GEN.	NUMBER OF INDIV- IDUALS OF PARENT IN FAMILY	NUMBER OF ♂♀	NUMBER OF ♀♀	NUMBER OF DEATHS	PER CENT OF ♂♀	PER CENT OF ♀♀	TYPE	REMARKS
	1911		1911								
1	Oct. 24	1		1	0	17	0	0	100	S	
2	Oct. 26	17	Oct. 26	1	0	17	0	0	100	S	
3	28	22	28	1	0	20	2	0	100	S	
4	30	21	30	1	4	17	0	19	81	S	
5	Nov. 1	21	Nov. 1	1	0	21	0	0	100	S	
6	2	23	2	1	4	19	0	17	83	S	
7	4	22	4	1	0	21	1	0	100	S	
8	6	6	6	1	0	6	0	0	100	S	
9	7	13	7	1	0	9	0	0	100	S	
10	9	12	9	2	0	6	6	0	100	S	
11	11	12	13	5	0	9	3	0	100	S	
12	17	3	17	1	0	3	0	0	100	S	
13	20	21	20	1	0	21	0	0	100	S	
14	22	10	22	1	0	9	1	0	100	S	
15	25	12	25	1	0	12	0	0	100	S	
16	27	8	29	6	0	5	3	0	100	S	
17	30	10	30	1	0	10	0	0	100	S	
18	Dec. 1	21	Dec. 1	1	2	18	1	10	90	S	Prod. H
19	3	21	3	1	0	21	0	0	100	S	
20	5	18	5	1	5	11	2	31	69	S	
21	6	6	6	1	0	6	0	0	100	S	
22	7	22	7	1	0	21	1	0	100	S	
23	8	4	8	1	0	4	0	0	100	S	
24	10	14	10	1	0	13	1	0	100	S	

[illegible]

TABLE 1 (Continued)

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOW- ING GEN.	NUMBER OF INDIVID- UALS OF PARENT IN FAMILY	NUMBER OF ♂'s	NUMBER OF ♀♀	NUMBER OF DEATHS	PERCENT OF ♂'s	PERCENT OF ♀♀	TYPE	REMARKS
	1912		1912								
54	26	16	27	2	0	15	1	0	100	S	
55	28	20	28	3	0	20	0	0	100	S	
56	30	16	30	1	5	11	0	31	69	S	
57	31	9	31	2	3	6	0	33	67	S	
58	Feb. 2	18	Feb. 2	1	6	11	1	32	68	S	
59	3	19	4	2	3	15	1	17	83	S	
60	5	22	5	1	2	20	0	9	91	S	Prod. H
61	7	18	8	4	9	9	0	50	50	S, I	Prod. H
62	10	16	10	1	2	14	0	13	87	S, I	
63	12	5	12	1	0	5	0	0	100	C	Ate Y
64	14	7	14	1	0	5	2	0	100	C	Ate Y
65	17	7	17	1	1	5	1	16	84	C, S	
66	18	8	18	1	0	8	0	0	100	H, S	
67	20	20	20	1	0	19	1	0	100	S	
68	21	20	21	1	4	16	0	20	80	S	
69	23	21	23	1	5	16	0	24	76	S	
70	25	13	25	3	0	11	2	0	100	S	
71	27	6	27	1	0	6	0	0	100	S	
72	29	20	29	1	0	19	1	0	100	S	
73	Mar. 2	10	Mar. 2	1	3	7	0	30	70	S	
74	3	22	3	1	4	18	0	18	82	S	
75	5	19	5	1	4	14	1	22	78	S	
76	7	16	7	1	0	15	1	0	100	S	
77	9	20	9	1	0	19	1	0	100	S	

131	28	2	28	1	0	2	0	0	100	S	Rest Disc.
132	29	4	29	1	0	3	1	0	100	S	
133	30	15	30	3	0	15	0	0	100	S	
134	31	13	31	1	0	13	0	0	100	S	
135	June 1	10	June 1	2	0	10	0	0	100	S	
136	2	10	2	2	0	9	1	0	100	S	
137	3	1	3	1	0	1	0	0	100	S	
138	4	9	4	1	0	9	0	0	100	S	Rest Disc.
139	5	8	5	1	0	8	0	0	100	S	Rest Disc.
140	7	15	7	2	0	14	1	0	100	S	Rest Disc.
141	8	9	8	2	0	8	1	0	100	S	
142	10	10	10	1	2	8	0	20	80	S	
143	11	7	12	2	1	6	0	14	86	S	
144	13	3	13	1	0	3	0	0	100	S	
145	14									S	Disc.

History of Series I

	1911		1911		1911		1911		1911		1911	
	Oct. 24	26	Oct. 26	28	Nov. 1	2	Oct. 26	28	Nov. 1	2	Oct. 26	28
1			1				0				0	
2		18	18	21			1	1	17	1	5	95
3		28		18			0	0	20	1	0	100
4		30		18			0	0	17	1	0	100
5	Nov. 1	18		18			0	0	17	1	0	100
6	2	15		15			2	13	13	0	12	88
	4	24		24			0	23	23	1	0	100
8	6	13		13			1	1	12	1	0	100
9	8	16		16			0	0	12	4	0	100
10	10	12		12			0	0	8	4	0	100
11	4	8		8			0	0	8	0	0	100
12	18	5		5			0	0	5	0	0	100

TABLE 1—Continued

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOW- ING GEN.	NUMBER OF INDIVID- UALS OF PARENT IN FAMILY	NUMBER OF ♂♀	NUMBER OF ♀♀	NUMBER OF DEATHS	PER CENT OF ♂♀	PER CENT OF ♀♀	TYPE	REMARKS
13	1911 Nov. 20	18	1911 Nov. 20	1	0	17	1	0	100	S	
14	21	41	25	11	0	8	3	0	100	S	
15	26	3	27	2	0	2	1	0	100	S	
16	28	10	29	2	0	9	1	0	100	S	
17	Dec. 1	14	Dec. 2	1	1	13	0	7	93	S	
18	2	22	1	1	1	21	0	5	95	S	Sis. Pro. H.
19	4	23	4	1	1	21	1	5	95	S	Sis. Pro. H.
20	5	20	5	1	0	20	0	0	100	S	Sis. Pro. H.
21	7	19	7	1	1	18	0	5	95	S	Sis. Pro. H.
22	8	20	8	2	0	20	0	0	100	S	Sis. Pro. H.
23	9	12	9	1	0	11	1	0	100	S	Sis. Pro. H.
24	10	20	10	1	0	20	0	0	100	S	
25	12	10	12	1	2	8	0	20	80	S	
26	14	23	14	1	0	23	0	0	100	S	
27	15	21	15	1	0	20	1	0	100	S	
28	16	15	17	3	0	13	2	0	100	S	
29	18	7	18	1	0	7	0	0	100	S	
30	20	9	20	1	0	9	0	0	100	S	
31	22	5	22	1	0	5	0	0	100	S	
32	24	13	25	5	0	10	3	0	100	S	
33	27	19	27	1	0	18	1	0	100	S	
34	29	18	29	2	0	17	1	0	100	S	
35 *	31	8	1912 Jan. 1	2	0	5	3	0	100	S	

TABLE 1—Continued

NUMBER OF 3RN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF INDIVIDUALS OF PARENT IN FAMILY	NUMBER OF ♂♀	NUMBER OF ♀♀	NUMBER OF DEATHS	PER CENT OF ♂♀	PER CENT OF ♀♀	TYPE	REMARKS
	1912		1912								
65	Feb. 17	20	Feb. 18	1	0	17	3	0	100	S	
66	20	13	20	1	0	10	3	0	100	S	
67	21	16	21	1	0	16	1	0	100	S	
68	23	9	24	3	0	9	0	0	100	S	
69	26	3	26	2	0	3	0	0	100	S	
70	28	21	29	5	0	19	2	0	100	S	
71	Mar. 1	20	Mar. 2	2	2	18	0	10	90	S	
72	3	20	3	1	0	19	1	0	100	S	
73	3	19	6	5	0	18	1	0	100	S	
74	8	2	8	2	4	16	1	20	80	S	
75	10	8	10	1	0	6	2	0	100	S	
76	12	6	12	1	0	6	0	0	100	S	
77	13	22	13	1	1	20	1	5	95	S	
78	15	15	15	1	0	15	0	0	100	S	
79	16	20	16	1	0	19	1	0	100	S	
80	18	22			0	21	1	0	100	S	Disc.
<i>History of Series J</i>											
	1911		1911								
1	Dec. 2	.1		2	1	2	6	33	67	S	Prod. H
2	4	9	Dec. 4	1	0	5	1	0	100	H	
3	6	6	6	1	2	16	0	11	89	H	
4	8	18	8	1	1	17	0	6	94	H	
5	9	18	10	4	1	17	0	0	100	I	
6	12	10	12	1	0	10	0	0	100	I	

4	Dec. 8	20	Dec. 8	1	2	12	6	14	86	H
5	9	15	9	1	0	15	0	0	100	H
6	11	20	11	1	3	15	2	17	83	H, I
7	13	13	14	5	0	13	0	0	100	I, H
8	15	20	16	2	0	20	0	0	100	H
9	17	20	17	1	0	20	0	0	100	H
10	19	16	19	1	0	16	0	0	100	H
11	20	17	20	1	1	16	0	6	94	H
12	22	12	22	2	0	10	2	0	100	H
13	24	6	24	1	0	6	0	0	100	I
14	27	15	27	2	0	14	1	0	100	S
15	29	20	29	2	0	19	1	0	100	H
16	31	17	31	1	0	17	0	0	100	H
17	1912 Jan. 2	17	1912 Jan. 2	1	3	14	0	18	82	H
18	3	15	3	1	0	15	0	0	100	H
19	5	15	5	1	0	12	3	0	100	H
20	6	14	6	1	0	14	0	0	100	H
21	8	14	8	1	0	14	0	0	100	H
22	9	14	9	1	0	14	0	0	100	H
23	11	13	11	2	0	13	0	0	100	H
24	13	11	13	1	1	10	0	9	91	H
25	15	5	15	2	1	4	0	20	80	H
26	16	12	16	1	2	10	0	17	83	H
27	17	20	17	2	1	18	1	5	95	H
28	19	22	19	1	3	19	0	16	84	H
29	21	14	21	1	0	14	0	0	100	H
30	23	12	23	1	0	12	0	0	100	H
31	24	12	24	1	0	12	0	0	100	H
32	25	10	25	1	0	9	1	0	100	H

TABLE 1—Continued

NUMBER OF GEN. IN SERIES—	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOW- ING GEN.	NUMBER OF INDIV- IDUALS OF PARENT IN FAMILY	NUMBER OF ♂♀	NUMBER OF ♀♀	NUMBER OF DEATHS	PER CENT OF ♂♀	PER CENT OF ♀♀	TYPE	REMARKS
	1912		1912								
33	Jan. 27	8	Jan. 27	1	5	3	0	63	37	H	
34	28	16	28	1	6	10	0	37	63	H	
35	30	16	30	1	10	6	0	63	37	H	
36	Feb. 1	21	Feb. 1	1	5	15	1	25	75	H	
37	4	3	4	1	2	1	0	66	33	H	
38	6	15	6	1	0	14	1	0	100	H	
39	8	20	9	3	2	18	0	0	100	H	
40	10	23	10	1	1	19	3	5	95	H	
41	12	8	12	3	2	14	2	12	88	H	
42	14	10	15	4	4	6	0	40	60	H	
43	17	8	17	1	5	3	0	62	37	H	
44	18	15	18	1	0	15	0	0	100	H	
45	20	10	21	3	5	4	1	55	45	H	
46	23	8	23	1	0	6	2	0	100	H	
47	25	16	26	3	4	11	1	26	74	H	
48	28	21	28	1	16	5	0	76	24	H	
49	Mar. 1	17	Mar. 1	2	7	9	1	44	56	H	
50	3	18	3	2	10	5	3	67	33	H	
51	5	12	5	1	4	8	0	33	67	H	
52	7	10	7	1	0	9	1	0	100	H	
53	9	13	9	2	5	8	0	39	61	H, I	
54	11	8	11	1	2	5	1	29	71	H	
55	13	21	13	1	6	15	0	24	76	S, H	
56	14	18	16	8	9	8	1	53	47	S, I, H	
57	17	20	17	1	10	10	0	50	50	H	

[illegible]

The histories of *J* and *K*, on the other hand, are very different and were a great surprise to us at the time the experiment was conducted. The transition from the saccate to the humped type which was so tardy and transient in *H* and *I*, appeared in *K* promptly in the second generation and in *J* in the third. Moreover, when once produced the humped type became singularly constant throughout almost the entire series. But once in *J* and three times in *K* did single generations occur in which a part of the individuals only reverted to the saccate type; yet, though no preference was shown in the selection of humped individuals to continue the series at these times of reversion and although no change of food was made, the humped type was immediately resumed in the next generation.

This constancy of the higher type was in striking contrast to our entire previous experience in the rearing of pedigree series of this rotifer. While in mass culture the humped type is readily maintained yet in no other instance of a long continued series of isolation cultures had it been possible to avoid frequent reversions to the smaller saccate type. The only instance where this was done for a score or more generations² was where a special food stimulus was applied. That our success with *J* and *K* was not due to food supply, employed is proven by the fact, which we have already suggested, that the copious food supply was taken from the same source as was that fed to *H* and *I* and was given to all in excess of consumption. In size *J* and *K* were of course larger than *H* and *I*, being of the humped type; the average number of young produced per individual approximated closely to fourteen in *J* and to fifteen in *K*.

A study of the metabolic rhythm of the four series, which may be made out by noting the number of young present in successive generations, shows the relative independence of the four series in regard to each other, and also in regard to external conditions. Their crescendos and diminuendos are not coincident. Nevertheless, a closer approach to parallelism obtains between *J* and *K*, on the one hand, and between *H* and *I*, on the other, than obtains between any two of the contrasted series.

² See article I, table 10, history of Series *D*.

In order to test further the nature of *H* and *I* in regard to their latent potentiality to produce the higher type, derivative mass cultures were started from time to time, it having been proven by all our previous experience that mass cultures favor the progressive mutation to the larger types. Irregular food supplies were purposely resorted to in these cultures as favorable to the transition. Transitions did indeed occur to the humped type, and in a few instances cannibalistic campanulates also made their appearance. This latter transition, however, was far less frequent than in parallel mass cultures derived from *J* and *K*.

The natural conclusion suggested by the conduct of the above series is that we do have the transmission of experimentally induced factors through the sexual gametes and the resulting resting egg. While this result is not shown morphologically in the generation immediately derived from the zygote yet it would seem to plainly exist as an inherent factor, the factor which we have termed the physiological potential.

In order to accumulate further evidence for the fact of this transmission and also to ascertain whether such transmission is, as it were, a uniform process or whether it may perhaps be variable or cumulative, or regressive, we continued the rearing of further series derived from resting eggs produced by the same stocks.

As the labor of rearing these stocks is very considerable and we wished to mass abundant proof upon the points tested we choose to limit the work to inbred series only.

Series derived by inbreeding individuals of I and J

It may be first explained that owing to the diverse inherent tendencies of reproduction it was easy to obtain resting eggs from humped series like *J* while it was a matter of much difficulty to obtain them from saccate series like *I*. It was thus not feasible to begin a new parallel series of these two types simultaneously. Adequate controls however were always conducted. To test the inheritance of the high potential of *J* new series were derived as follows: On January 15, during the twenty-first generation of *J*, a mass culture was obtained from the fourteenth daughter of the

mother in the pedigree line. This culture was treated according to our regular method for inducing male production.³ The effect was quite successful. Resting eggs followed. On January 20 sister eggs from one individual were isolated under conditions favorable for hatching, which began by February 1. Ten of these young were isolated in cultures normal as to food supply and to all other known conditions. These ten individuals became the parents of ten series which, for brevity, we designate collectively as *J 2*.

The effort was made to parallel *J 2* with series derived from the series *H* or *I*, but as already mentioned, difficulty is always expe-

³ In an article appearing in *Science*, vol. 38, pp. 786-788, A. Franklin Shull, in discussing this method of determining sex or male production, offers certain objections based upon his study of *Hydatina senta*. In regard to the relation of physiological rhythm, nutrition and male production, we should state that since the last two may be directly controlled or modified by experimental conditions, and since the first may also be modified, though not by direct methods, we have a means whereby we may govern this relation at will. That Shull has healthy lines which pass through long periods of parthenogenetic female production, is not surprising. We have had parallel cases; for instance, one of our healthy lines had given rise to a continuous parthenogenetic female production for over sixty generations. However, conditions were clearly not such that male production was possible, but later when conditions were altered to those favorable to male production, this hitherto total female-producing line suddenly threw a large percentage of male producers. That this was due entirely to some unknown internal factor would seem all but improbable. But even accepting this, would it help in explaining results which have been obtained?

In our study of sex determination we have attempted to emphasize that the individual is the "point of action" rather than a number of generations. A propos to this we would call attention to those cytological facts obtained by Erlanger and Lauterborn (*R. Zool. Anz.*, Bd. 20), Lensen (*Zool. Anz.*, Bd. 21), and Jennings (*Bull. Mus. Comp. Zool.*, Harvard Coll., No. 30), upon *Hydatina* and *Asplancha* which tend to prove that during the period of maturation sex is determined by the casting off of the polar bodies. The reduction in the number of chromosomes occurring when the second polar body is cast off, results in male production, while in ova which cast off only one polar body there results female production. The time at which this maturation occurs corresponds to the time at which the factors we advocate are active in the determination of sex. It would be rather difficult to correlate this evidence, it seems to us, with definite internal causes or to chemicals which act only upon the preceding generation, as is offered in explanation of sex determination by Shull.

rienced in obtaining resting eggs from the saccate type. However, a mass culture started on January 29, from the fifty-third generation of *I* and remaining purely saccate, produced by February 6 resting eggs. These began to hatch by February 12. Unfortunately, at this time the labor of caring for the enormous progeny of the ten lines of *J 2*, as well as of the original series, rendered it impossible to establish more than a single additional series. This, started from one of the before-mentioned resting eggs drawn from the saccate series, *I*, we term series *I 2*. It thus forms a parallel or contrast series to the ten lines in *J 2*. Other individuals derived from sister eggs, as well as from other resting eggs produced at the same time in the same culture, were set aside to form mass cultures useful in a general way as checks or controls. The history of *J 2* as well as of the contrasted series *I 2* are given in table 2.

It will be seen from table 2 that seven of the series in *J 2* were bred for ten generations, the other three succumbing to total male production after six to eight generations. *I 2* was bred to thirty generations.

It will be noted that again sexual reproduction fails to wipe out the induced but hereditary tendencies of the contrasted series. Although conditions were in every way parallel the transition from the saccate to the humped type occurred early in each of the ten lines of *J 2*, while in *I 2* no transition whatever occurred. In reality the contrast is even stronger than indicated by this general statement, for not only is there no tendency toward mutation displayed during the entire thirty generations of *I 2*, but in not one of the above mentioned mass cultures derived from the resting eggs of the same source did the humped type appear. The exact generation, on the other hand, in which the humped type appeared in the lines of *J 2* were, respectively, the ninth, fifth second, third, third, third, fourth, sixth, fourth and third. It is noteworthy that even in the one instance where the transition in the direct line was delayed till the ninth generation sister individuals to the one in the direct line made the transition as early as the third generation.

TABLE 2
History of Series J 2, derived from mass culture of 14 individual of J. Humped parents
No. 1

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOWING GEN.	NUMBER OF INDIVIDUALS OF PARENT IN FAMILY	NUMBER OF ♂♂	NUMBER OF ♀♀	NUMBER OF DEATHS	PER CENT OF ♂♂	PER CENT OF ♀♀	TYPE	REMARKS
1	1912 Feb. 1	1	1912 Feb. 1	1	0	11	0	0	100	S	Sis. Prod. H
2	3	11	3	3	0	6	2	0	100	S	
3	5	8	5	2	0	3	0	0	100	S	
4	8	3	8	1	0	3	0	0	100	S	
5	9	11	10	2	0	11	0	0	100	S	
6	11	11	12	4	1	9	1	10	90	S	
7	14	7	14	2	3	4	0	43	57	S	
8	16	8	16	1	1	7	0	12	87	S	
9	19	8	19	3	3	4	1	43	57	H	
10	21	6			0	6	0	0	100	H	

No. 2

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOWING GEN.	NUMBER OF INDIVIDUALS OF PARENT IN FAMILY	NUMBER OF ♂♂	NUMBER OF ♀♀	NUMBER OF DEATHS	PER CENT OF ♂♂	PER CENT OF ♀♀	TYPE	REMARKS
1	1912 Feb. 1	1	1912 Feb. 3	1	0	1	1	0	100	S	
2	3	2	3	1	0	3	0	0	100	S	
3	5	3	5	1	0	3	1	57	43	S	
4	7	8	8	4	4	9	0	40	60	H	
5	10	15	10	1	6	9	0	0	100	H	
6	12	9	13	2	0	9	0	0	100	H	
7	14	8	14	1	0	8	0	0	100	H	
8	16	8	16	1	1	6	1	14	86	H	
9	18	14	18	2	1	12	1	8	92	H	
10	19	9			5	3	1	68	32	H	

		1912		1912		No. 3			
	Feb.	1	3	Feb.	5	4	3	10	0
1				1					
2			13	13		7	7	5	0
3		8		12	11	7		7	0
4		13		8	13	1	1	7	0
5		15		11	16	2	1	10	0
6		18		11	18	1	1	10	0
7		20		17	20	1	8	8	1
8		22		10	22	1	1	7	2
9		24		9	24	1	2	6	1
10		26		7			0	6	1

[illegible]

1912		1912		No. 5	
	Feb.		Feb.		
1	2	1	4	0	3
2	4			1	0
3	7	4	7	0	0
				0	100
				0	100

TABLE 2—Continued
No. 5—Continued

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOWING GEN.	NUMBER OF INDIVIDUALS OF PARENTIN FAMILY	NUMBER OF ♂♂	NUMBER OF ♀♀	NUMBER OF DEATHS	PER CENT OF ♂♂	PER CENT OF ♀♀	TYPE	REMARKS
No. 5											
4	1912 Feb. 8	11	1912 Feb. 10	6	6	5	0	54	46	H	
5	12	10	12	2	0	10	0	0	100	H	
6	14	12	14	1	1	11	0	9	91	H	
7	15	16	16	2	10	6	0	63	37	H	
8	18	12	21	8	7	4	1	64	36	H	
9	23	10	23	1	1	9	0	10	90	H	
10	25	8			0	7	1	0	100	H	
No. 6											
1	1912 Feb. 2	1	1912 Feb. 5	2	1	8	0	12	87	S	Sis Prod. H
2	4	9	8	1	1	3	1	25	75	H	
3	8	5									
4	19	12	19	2	0	11	1	0	100	H	
5	13	6	13	1	2	4	0	33	67	H	
6	15	7	16	3	5	2	0	72	28	H	
7	18	8			7	1	0	87	13	H	
No. 7											
1	1912 Feb. 2	1	1912 Feb. 6	1	0	3	2	0	100	S	
2	6	5									
3	8	6	9	4	0	4	2	0	100	S	
4	11	3	11	1	0	2	1	0	100	H	

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9 0 0 100

2 0 0 0

10 6 2 0

1 0 0 7

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TABLE 2—Continued
No. 10

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOW- ING GEN.	NUMBER OF INDIVID- UALS OF PARENT IN FAMILY	NUMBER OF ♂♀	NUMBER OF DEATHS	PER CENT. OF ♂♀	PER CENT. OF ♀♀	TYPE	REMARKS
1912										
1	Feb. 6	1								
2	Feb. 9	6		1	0	6	0	100	S	
3	12	6		1	2	4	0	33	H	
4	14	13		1	2	11	0	15	H	
5	16	10		3	1	8	1	11	H	
6	18	5			3	0	2	100	H	
History of Series 12, derived from mass culture from the fifty-third generation of I. Parents of sacate type										
1913										
1	Feb. 12	1								
2	Feb. 17	5		1	0	5	0	100	S	
3	18	17		3	0	15	2	100	S	
4	21	6		1	0	6	0	100	S	
5	23	4		1	0	3	1	100	S	
6	25	5		2	0	5	0	100	S	
7	28	19		1	0	19	0	100	S	
8	Mar. 1	21		1	4	17	0	81	S	
9	2	9		1	0	8	1	100	S	
10	4	11		6	0	11	0	100	S	
11	8	5		1	0	5	0	100	S	
12	10	8		2	0	7	1	100	S	
13	12	8		1	0	6	2	100	S	
14	13	13		2	0	12	1	100	S	

It can not be other than surprising to find this all but uniform maintenance of high and low potential respectively in these series of rotifers, carried not only through long series of parthenogenetic reproduction, but through sexual reproduction as well, and this in spite of the fact that sexual reproduction involves the temporary return to the apparently uniform saccate type.

To test the matter still further, through more cycles of sexual reproduction, and also to make up for the paucity of numbers in the series on the saccate side in our last experiment, further experiments were begun as follows: Mass cultures were founded from the tenth and fourteenth generations of the saccate series *I 2*. These were carefully fed so as to induce male production without the customary accompaniment of the transition to the humped type. This end was successfully attained in both cultures. A young female from the mass culture derived from the tenth generation was fertilized, and by March 20 had produced resting eggs. Two of these hatched on March 27, one of which became the parent of a pedigree series termed *1 of I 3*. From the mass culture derived from the fourteenth generation of *I 2* more resting eggs were secured. A number were dropped by four individuals on March 12 and 22. These were isolated and from a large number which hatched on March 28 and 29 fourteen were chosen to become the parents of pedigree series, which make up the second part of the group of series termed *I 3*. This group of series thus compose the third group of inbred saccate lines derived by sexual reproduction, each group from the preceding group, and all from the original parthenogenetic individual.

That these series of *I 3* might be paralleled by at least a few series derived from our humped lines two new series were started from *J*. It was perhaps unfortunate that these series could not have been started from some one of the ten series of *J 2*, but these series had been discarded after the end of the tenth generation, as we had not at that time considered the possibility of the prolonged continuence of our experiments. It thus became necessary to have recourse to the original series *J*. On March 15 the tenth individual of the fiftieth generation of *J* was fertilized by a male produced by a sister individual of the same generation. Resting eggs were formed which hatched on March 26. One only was

saved and from it was reared a series which we term *J 2'*. Another series of similar derivation was reared from a resting egg produced by the third individual of the fifty-fourth generation, fertilized by a male, a brother to the one used to fertilize the mother of the preceding series. The series was begun by isolating the individual on March 24. We term this series *J 2''*. The history of the fifteen series of *I 3*, together with the contrasting series *J 2'* and *J 2''*, is given in table 3.

The study of table 3 shows again the surprising fact—the complete hereditary continuence through the resting egg of the diverse qualities originally induced in the two contrasting stocks. Throughout the entire ten generations through which the fifteen series of *I 3* were carried, not a single instance of the transition from the saccate type to the humped type occurred, although in a few of numerous mass cultures derived from these saccate series such transitions did occur, though sparingly. On the other hand, despite uniform feeding and other parallel conditions, the lines *J 2'* and *J 2''* again leapt almost immediately to the humped form. This occurred in the second and third generation respectively, and it is of no less significance that there was in each of these series an almost immediate production, no doubt through cannibalism, of the third or campanulate type. This shows still more conclusively the inherent high potential so fully maintained by this stock.

Although no farther evidence was needed to test the single matter of inherited potential through the resting egg, yet the thought of the possibility of the formation of a saccate race devoid of any mutational tendency induced us to develop still more series. A culture derived from the fourth generation of series *11* of *I 3* was allowed to mass. Males were produced by saccate individuals on April 14 and resting eggs were duly formed by saccate females. An individual, hatched from one of these, gave the starting point of series *I 4*. It was bred through thirteen generations. Its history is given in table 4. Again the saccate type was constant throughout the entire series. But in mass cultures, which were now forced by special feeding to test at once their character, it was still possible to induce the transformation to the humped type. No campanulates were formed.

TABLE 3

History of Series I 3, derived from mass culture from the tenth generation of I 2. Parents of sacate type. No. 1

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOWING GEN.	NUMBER OF INDIVIDUALS OF PARENT IN FAMILY	NUMBER OF ♂♂	NUMBER OF DEATHS	PERCENT OF ♂♂	PERCENT OF ♀♀	TYPE	REMARKS
1	1912 Mar. 27	1	1912 Mar. 27	1						
2	29	10		2	0	9	0	100	S	
3	31	13	31	1	3	8	28	72	S	
4	Apr. 2	14	Apr. 2	1	0	13	1	100	S	
5	3	13	3	1	0	12	1	100	S	
6	4	18	4	1	0	17	1	100	S	
7	5	16	5	1	8	8	0	50	S	
8	6	12	6	1	1	10	1	91	S	Disc.
9	8	4	8	2	0	4	0	100	S	Disc.
10	10	22			0	20	2	100	S	

Derived from mass culture from the fourteenth generation of I 2. Parents of sacate type. No. 2

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOWING GEN.	NUMBER OF INDIVIDUALS OF PARENT IN FAMILY	NUMBER OF ♂♂	NUMBER OF DEATHS	PERCENT OF ♂♂	PERCENT OF ♀♀	TYPE	REMARKS
1	1912 Mar. 29	1	1912 Mar. 30	1						
2	30	19		1	1	18	0	95	S	
3	31	18	31	1	0	18	0	100	S	
4	Apr. 2	10	Apr. 2	1	0	10	0	100	S	
5	4	17	4	1	0	16	1	100	S	
6	5	18	5	1	2	16	0	89	S	
7	6	7	6	1	2	5	0	72	S	
8	7	13	7	1	0	13	0	100	S	
9	8	28	8	1	1	27	0	64	S	
10	10	7			0	6	1	100	S	

No. 3									
1912									
1	Mar. 29	1							
2	Mar. 30	14	Mar. 29	1	0	14	0	100	S
3	Apr. 1	16	Apr. 1	1	0	13	3	0	100
4	Apr. 3	18	Apr. 3	1	7	10	1	41	59
5	Apr. 4	15	Apr. 4	1	3	12	0	20	80
6	Apr. 5	22	Apr. 5	3	5	16	1	24	76
7	Apr. 6	21	Apr. 6	1	6	13	2	32	68
8	Apr. 7	20	Apr. 8	3	4	16	0	20	80
9	Apr. 9	23	Apr. 9	1	11	12	0	48	52
10	Apr. 10	21			3	18	0	15	85
No. 4									
1912									
1	Mar. 29	1							
2	Mar. 30	16	Mar. 30	1	3	13	0	19	81
3	Mar. 31	8	Mar. 31	1	0	7	1	0	100
4	Apr. 2	22	Apr. 2	1	2	18	2	10	90
5	Apr. 3	11	Apr. 3	1	2	9	0	18	82
6	Apr. 4	25	Apr. 4	1	11	13	1	46	54
7	Apr. 5	20	Apr. 5	1	5	15	0	25	75
8	Apr. 6	18	Apr. 6	1	1	17	0	6	94
9	Apr. 8	18	Apr. 8	1	1	16	1	6	94
10	Apr. 10	23			7	16	0	30	70
No. 5									
1912									
1	Mar. 29	1							
2	Mar. 31	6	Mar. 31	1	0	4	2	0	100
3	Apr. 1	14	Apr. 1	1	0	13	1	0	100

TABLE 3—Continued

No. 5—Continued

NUMBER OF ORG. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOW- ING GEN.	NUMBER OF INDIV- IDUALS OF PARENTIN FAMILY	NUMBER OF ♂	NUMBER OF ♀	NUMBER OF DEATHS	PER CENT OF ♂	PER CENT OF ♀	TYPE	REMARKS
4	1912 Apr. 4	11	1912 Apr. 4	1	2	9	0	18	82	S	
5	5	7	5	1	0	7	0	0	100	S	
6	6	5	6	1	1	4	0	20	80	S	
7	8	3	8	1	2	1	0	67	33	S, I	
8	9	8	9	1	4	4	0	50	50	I, S	
9	10	21	10	1	0	21	0	0	100	S	
10	11	20	11	1	0	20	0	0	100	S	

No. 6

	1912 Mar. 29	1912 Mar. 30	1912 Apr. 1	1912 Apr. 2	1912 Apr. 4	1912 Apr. 5	1912 Apr. 7	1912 Apr. 8	1912 Apr. 9	1912 Apr. 11
1	1	4	18	7	17	13	5	4	4	19
2	4	1	2	4	5	6	8	9	11	17
3	1	2	4	5	6	8	9	11	17	17
4	2	4	5	6	8	9	11	17	17	17
5	4	5	6	8	9	11	17	17	17	17
6	5	6	8	9	11	17	17	17	17	17
7	6	8	9	11	17	17	17	17	17	17
8	8	9	11	17	17	17	17	17	17	17
9	9	11	17	17	17	17	17	17	17	17
10	11	17	17	17	17	17	17	17	17	17

Rest disc.
Rest disc.

No. 7

1912

1912

	1912	1912
1	Mar. 29	1
2	Mar. 30	16
3	Mar. 31	17
4	Apr. 4	15
5	Apr. 5	21
6	Apr. 6	24
7	Apr. 8	21
8	Apr. 10	13
9	Apr. 11	18
10	Apr. 12	20

1	15	0	6	94	z
0	17	0	0	100	z
1	13	1	7	93	z
5	16	0	24	76	z
0	23	1	0	100	z
8	13	0	38	62	z
6	7	0	46	54	z
1	16	1	6	94	z
2	18	0	10	90	z

No. 8

1912

1912

	1912	1912
1	Mar. 29	1
2	Mar. 30	26
3	Mar. 31	14
4	Apr. 2	11
5	Apr. 3	23
6	Apr. 5	13
7	Apr. 6	9
8	Apr. 8	4
9	Apr. 10	22
10	Apr. 11	17

1	26	0	0	100	z
0	12	2	0	100	z
1	10	0	9	91	z
5	18	0	22	78	z
4	9	0	30	70	S, I
0	8	1	0	100	1, K
1	3	0	25	75	S
2	21	0	5	95	S
1	15	2	0	100	z

No. 9

1912

1912

	1912	1912
1	Mar. 29	1
2	Mar. 30	18
3	Mar. 31	12

1	17	0	6	94	S
3	5	4	37	62	S

No. 9—Continued

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOUNDED BY LIVING GEN.	NUMBER OF INDIVID- UALS OF PARENT IN FAMILY	NUMBER OF ♂'s	NUMBER OF ♀'s	NUMBER OF DEATHS	PERCENT OF ♂'s	PERCENT OF ♀'s	TYPE	REMARKS
	1912		1912								Rest disc.
4	Apr. 2	6	Apr. 2	1	0	6	0	0	100	S	
5	4	20	5	5	5	13	2	28	72	S	
6	6	20	6	1	0	19	1	0	100	S	
7	8	22	8	1	5	16	1	24	76	S	
8	9	12	10	4	2	10	0	17	83	S	
9	11	23	11	1	6	16	1	27	73	S	
10	12	22		0	0	22	0	0	100	S	

[illegible]

[illegible]

No. 12									
1912			1912						
1	Mar. 28	1							
2	Mar. 30	5	1	0	5	0	0	100	S
3	Mar. 31	16	1	1	15	0	6	94	S
4	Apr. 1	8	4	0	6	2	0	100	S
5	Apr. 4	8	1	4	4	0	50	50	S
6	Apr. 5	24	1	8	14	2	36	64	S
7	Apr. 6	5	3	2	3	0	40	60	S, I
8	Apr. 9	22	1	2	20	0	10	90	I, S
9	Apr. 10	22		3	17	2	15	85	S
Rest disc.									
Rest disc.									

		1912		No. 13					
1	Mar. 28	1							
2	30	5							
3	31	10		1	0	4	1	0	100
				1	0	10	0	0	100

TABLE 3—Continued

No. 13—Continued

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOW- ING GEN.	NUMBER OF INDIVID- UALS OF PARENT IN FAMILY	NUMBER OF ♂'s	NUMBER OF ♀'s	NUMBER OF DEATHS	PERCENT OF ♂'s	PERCENT OF ♀'s	TYPE	REMARKS
	1912		1913								
4	Apr. 1	20	Apr. 1	1	5	14	1	26	74	S	
5	Apr. 2	12	2	1	1	11	0	9	91	S	
6	4	16	4	1	0	16	0	0	100	S	
7	5	19	5	1	3	16	0	16	84	S	
8	6	21	6	1	14	7	0	67	33	S	
9	8	23	8	1	0	23	0	0	100	S	
10	9	22			0	20	2	0	100	S	

No. 14

	1912		1913								
1	Mar. 28	1	Mar. 30	1	2	6	2	25	75	S	
2	30	10	31	1	1	7	0	12	88	S	
3	31	8			6	13	0	32	68	S	
4	Apr. 2	19	Apr. 2	1	9	8	0	53	47	S	
5	3	17	3	1	7	10	1	41	59	S	
6	4	18	4	1	0	2	0	0	100	S	
7	5	2	5	1	1	15	0	6	94	S	Rest disc.
8	6	16	6	1	1	12	0	14	86	S	
9	8	14	8	1	2	13	0	23	77	S	
10	9	17	9	1	4	13	0			S	

No. 15

1911										1912									
1	Mar. 28	1								1	2	17	•	1	11	89	S		
2	Mar. 29	20								1	0	19	0	0	0	100	S		
3	Mar. 30	19								1	5	21	0	0	24	76	S		
4	Apr. 1	26								1	2	17	0	0	12	88	S		
5	Apr. 2	19								1	1	9	0	0	10	90	S		
6	Apr. 3	10								1	14	6	0	0	70	30	S		
7	Apr. 4	5								1	3	12	0	0	20	80	S		
8	Apr. 5	6								1	9	10	0	0	47	53	S		
9	Apr. 6	15								1	4	13	2	2	23	77	S		
10	Apr. 7	19																	

History of Series J', derived from tenth individual of the fiftieth generation of J.										Parents of humped type.									
1912										1912									
1	Mar. 25	1								4	3	4	0	0	43	57	I,C,H		
2	Mar. 27	7								1	0	6	0	0	0	100	C		
3	Mar. 30	6								1	0	3	+	0	0	100	C,H		
4	Apr. 1	3								1	0	4	0	0	0	100	H,C		
5	Apr. 3	4								1	3	5	0	0	37	63	H		
6	Apr. 5	8								1	4	11	1	1	26	74	H		
7	Apr. 6	16								1	2	4	0	0	33	67	H,C		
8	Apr. 8	6								1	21	5	0	0	81	19	H		
9	Apr. 10	26								1	17	4	1	1	81	19	H		
10	Apr. 11	22																	

History of Series J 2''

1912									
1	Mar. 24	1							
2	Mar. 25	8							
3	Mar. 28	8							

S
H,C100
570
431
17
40
31
3

TABLE 2--Continued
History of Series J 2--Continued

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOW- ING GEN.	NUMBER OF INDIVID- UALS OF PARENT IN FAMILY	NUMBER OF ♂♂	NUMBER OF ♀♀	NUMBER OF DEATHS	PER CENT OF ♂♂	PER CENT OF ♀♀	TYPE	REMARKS
4	1912 Mar. 30	18	1912 Mar. 30	1	1	17	0	6	94	S, I	
5	31	14	31	1	3	11	0	21	79	H	
6	Apr. 2	25	Apr. 2	1	10	14	1	42	58	H	
7	4	18	4	1	10	6	2	63	37	H	
8	5	25	5	1	7	18	0	28	72	H	
9	7	22	7	1	14	8	0	64	36	H	
10	9	27			10	17	0	37	63	H	

TABLE 4
History of Series I 4, derived from a mass culture from the fourth generation of Series 12 of I 3. Parents of saccate type

NUMBER OF GEN. IN SERIES	DATE OF BIRTH OF FIRST YOUNG	NUMBER OF YOUNG	DATE OF BIRTH OF PARENT FOR FOLLOW- ING GEN.	NUMBER OF INDIV- IDUALS OF PARENT IN FAMILY	NUMBER OF ♂'s	NUMBER OF ♀'s	NUMBER OF DEATHS	PERCENT OF ♂'s	PERCENT OF ♀'s	TYPE	REMARKS
1	1912 Apr. 16	1	1912								
2	20	3	Apr. 16	1	0	3	0	0	100	S	
3	21	15	21	1	0	15	0	0	100	S	
4	23	15	23	2	0	15	0	0	100	S	
5	25	12	25	1	0	12	0	0	100	S	
6	26	8	26	3	0	8	0	0	100	S	Rest disc.
7	28	20	28	1	0	20	0	0	100	S	
8	30	18	30	1	0	16	1	6	94	S	
9	May 1	7	May 1	1	3	4	0	43	57	S	Rest disc.
10	2	14	2	1	0	14	0	0	100	S	
11	3	19	3	1	0	19	0	0	100	S	
12	4	2	4	1	0	2	0	0	100	Z	Rest disc.
13	5	20			0	19	1	0	100	S	

It was difficult to ascertain whether the tendency to remain in the saccate form was a cumulative one or whether it was remaining constant throughout the experiment. Perhaps the most obvious sign of the decreasing potential was the fact that it was becoming more difficult to produce male production in the saccate series.

In connection with male production, in *I 4*, there occurred, too, for the first time, an interesting phenomenon which points with some probability toward the possibility of a transition to a lower type than the ordinary saccate *A. amphora*. This was the production of a rather unusual number of males, among which a number appeared like transitional forms between the males of *A. amphora* and *A. brightwelli*. By isolating mothers about to produce males it was discovered that these unusual individuals were the early members born in male families; their humps were small or almost lacking, the body was much smaller and more compact than normal, and the corona was much narrowed. All these characters suggest the males of the smaller species. Transitional males were found in all stages between this much reduced type and the customary type of *A. amphora*.

One more series of saccates was bred, which we name *I 5*. It was started in the usual way by resting eggs secured from mass culture, which latter in its turn, had been derived from the seventh generation of *I 4*. This last series of saccate rotifers was bred for ten generations (table 5). It showed every evidence of possessing the full vigor of its predecessors and was likewise perfectly true to type so long as bred under the customary and constant conditions. A mass culture founded from the fifth generation failed entirely to throw the humped type. It again produced a small number of the transition-like males. Last of all, in the seventh and eighth generations, the series was itself again tested, by forced and alternate feeding, to see whether the potentiality of the humped type was still present. Mutation was indeed induced showing that the long hereditary transmission, sexual as well as asexual, of the simple saccate form had not produced a race sufficiently stable to resist the cause for change which lies in an unwonted food stimulus.

It is natural to ask whether any tendency toward such race formation had manifested itself throughout the entire succession

of series from *I 1* to *I 5*. If present, such tendency was weak and did not show itself in any phenomena capable of definite record, unless it be that the earlier mass cultures were a little more prone to undergo the upward transition than were those of the later series of *I*. It is our judgment also that it required more delicate manipulation of feeding technique to induce the transition at the close of our experiments than it did at the beginning. But too much weight should not be placed upon this judgment; its value is only suggestive.

More or less paralleling the last saccate series, *I 5*, two new races of humped stock were again reared, derived however in a somewhat different way, and calculated to test a new point. By April 19 the series *K* had reached the seventy-seventh generation, having produced, through a period of five months, an all but unbroken succession of humped generations. Suddenly in the seventy-seventh generation a single unusually small family occurred of but five individuals of which two were saccates, one transitional, and two of the prevailing type. We desired to test the hereditary qualities, sexual and asexual, of these accidental reversions, coming as they did in the midst of the strong humped series, *K*. Would they, after all, show the potential of their series or would it turn out that they had dropped permanently to the potential of *I*? One of the saccates was accordingly isolated as the progenitor first of a mass culture. Even this mass culture answered our main query by an almost immediate saltation to the humped form. And in less than a week there followed a second saltation to the campanulate and cannibalistic type. It was from some of these huge cannibals that males and young females were secured which produced resting eggs. Subjecting these to conditions favorable to hatching, two young were secured to become the parents of the series which, having the same point of origin, we group together and term *K 2*. They were bred for but ten and eleven generations respectively before being discarded. Their histories are given in table 6. A glance at this table shows that these series, like those derived from the humped line *J*, again rose to the humped condition in the earliest generations, that is, in the second and third, respectively. The rotifers of

TABLE 6

History of Series K 2, derived from mass culture from the seventy-seventh generation of K. Parents of campanulate type

[illegible]

No. 2

these lines were very large and showed much vigor. This last experiment with humped lines not only adds one more instance of unbroken heredity through the resting egg, but is of interest in that the stock was derived from series *K* rather than from *J*, showing that the two series comport themselves quite alike in this respect. The origin and conduct of the mass culture from which *K* 2 was derived is perhaps of still more interest. It shows that a single isolated parthenogenetic reversion to the saccate type does not necessarily mean a permanent drop in potential to the saccate level. Why, we can not say, but may easily conjecture that the isolated appearance of such saccates may have been due to a somatic rather than a germinal influence.

CONCLUSION

We need hardly repeat, in résumé, the main conclusion to which these experiments lead: The hereditary transmission of induced germinal modification through sexual as well as through parthenogenetic generations. Certain minor aspects of the matter, however, need further accentuation. First, the unique fact that a certain type or form when once induced (the humped form of *A. amphora*) may in reality be transmitted with full force despite an interruption of one or more generations (saccates from resting eggs) in which this form is not expressed. Although the conditions are totally different the phenomenon suggests that of recessive and dominant characters.

Second, it is worth while to accentuate the nature of the results of sexual reproduction in this rotifer with regard to vitality and variation. The limits of our experiments, it is true, are narrow, as all of our series were begun with inbreeding. But within these limits the results are definite and uniform. The union of gametes has little if any influence upon the vitality of the race; it is true that the resting eggs hatch irregularly but once hatched they show the normal growth and reproductive energy of the strain from which they are derived. There is no added vitality, no rejuvenation. As to variation again, the results of fertilization seem purely negative, excepting of course, the temporary return to

the saccate form already mentioned. In many respects the conduct of this rotifer when bred in pedigree series suggests parallelism with Protozoa under like handling. But the variation, so often degenerative, which Jennings has shown to result from conjugation in *Paramecium*, finds no parallel within our experiments, so far as we have carried them. On the contrary, sexual reproduction and parthenogenesis seem very closely related and all but equivalent processes.

We may perhaps note in this connection that we have observed a number of instances of simultaneous development of resting eggs and the active young males within the body of the same parent; and what is still more striking in these cases of mixed reproduction, certain of the earlier developing embryos were plainly covered with thin though characteristic coats resembling the resting egg. In some instances such embryos were proven to become females and thus undoubtedly represent fertilized eggs which because of special conditions—possibly the thinner egg coats—were proceeding to immediate development within the uterus quite as do the ordinary parthenogenetic ova. In any case the close relationship of the different types of reproduction was strongly called to mind by the observation of females bearing simultaneously the fully-formed resting eggs, active young males approaching the birth period, and the above mentioned intermediate type of reproductive body.

As to the bearing of these conclusions, it is plain that they throw much light upon our own previous experiments with this rotifer, and, we think, some as well upon the extended experiments of others upon the rotifer, *Hydatina senta*. Thus in our own previous work, entire lines of rotifers started from resting eggs chosen at random, often proved perplexingly dissimilar. They possessed what we now call different potentials, some tending strongly toward the maintenance of the saccate type, some toward the maintenance of the humped type, while others maintained this form plus the occasional production of the campanulate type. It is plain now, after the experiments recorded in this paper, that these contrasting lines of *A. amphora* may have differed solely because of a different parentage of the resting eggs

from which they were started. The frequent outburst, within certain lines, of the mutational tendency in the early generations, which we explained in our former paper as probably due to qualitative nutritional change, we now see may well be due to inherited high potential, with or without the special food stimulus.

Indirectly too, the question of the percentage of male production in different lines meets with a much needed additional explanation. Male production, as we showed in our previous paper, requires as its first and most fundamental condition, a state of high potential. It thus follows naturally that strains originating from resting eggs produced by parents in which this high potential had been built up by special nutrition, or had been inherited parthenogenetically, should become at once strains producing many males; whereas, conversely, strains from resting eggs dropped by parents of low potential inherit and maintain this same inner condition, and while perfectly healthy, produce under normal conditions few males.

In regard to the work of others upon the rotifer *Hydatina*, we find that Punnett, Whitney, and Shull, all in one way or another emphasize the fact of races or strains in their material. These strains of *Hydatina* show no morphological differences but contrast with each other in regard to number of males produced or in regard to the general rate of parthenogenetic reproduction. Both Shull and Whitney have also shown that the qualities of their strains may be transmitted through the resting egg. We believe, however, that in no case have they traced their strains to a definite origin or shown that any quality induced by a known agent became hereditary. Some of the work upon *Hydatina* produces the general impression of a species, split up into what one might be tempted to call 'elementary' strains, akin, in their fixity if not in their morphological differences, to Jennings' races of *Paramecia*; other portions of their work suggest, on the other hand, that these strains are less fixed and in some cases arise while the animals are under experimental observation. We hope that our work may throw some light upon the method of their origin, on their varying degrees of stability, and upon their frequent assumption of a hereditary character.

Lastly, we find in our results suggestions toward the explanation of many of the puzzling diversities of condition in which *Asplanchna amphora* and allied rotifers have been found in nature. We will not enter into details. But it is worth pointing out that some of the systematic students of the genus seem to have known only the saccate type; a number have now found, in at least feeble development, the rotifer in its dimorphic condition, that is, the saccate type giving rise to the humped form; while it seems doubtful at the present time whether the trimorphic condition, which is so copiously present in our vicinity, has as yet been met with elsewhere. After making full allowance for the different methods of study, and so forth, it seems highly probable that this rotifer and its immediate allies exist in nature in different degrees of development in different localities. Under the least favorable conditions it is possible that the saccate type only has been produced. Under more favorable conditions the potential has been raised and inheritable germinal variations have been induced, making possible the regular production of the humped type. Much more rarely, under conditions favoring the development of the species in unusual numbers, the striking though less fixed type which we call the campanulate has resulted. This type transmits its form, never to all, but always to a part of its offspring.

In the brief period of our experiments—about eight months—we were unable to produce types which were stable save when bred under approximately uniform conditions. Extreme starvation or sudden change in opulent nutrition would bring about a reversion to a simpler or stimulate to a higher type. In so far, our work falls short of offering conclusive evidence that the processes which we have described are related to species formation. Nevertheless it is easy to imagine that similar steps may now and then become more permanent in nature. In any case, it is interesting to know that the marked morphological changes induced by nutrition are not confined within the bounds of parthenogenetic series only, but, as far as our experiments show, are equally transmitted by the sexual process. This it seems to us, renders it at least not improbable that the mutational changes shown by this rotifer are phases of a true species-making process.

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CERTAIN RELATIONS BETWEEN RHEOTAXIS AND RESISTANCE TO POTASSIUM CYANIDE IN ISOPODA¹

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Earlier experiments (Allee '12-'13) showed that certain conditions known to affect the rate of metabolism of animals regularly affected the rheotactic reaction of the isopod, *Asellus communis*, Say. Low oxygen, chloretone, potassium cyanide, lowered temperature, suddenly heightened temperature, increased carbon dioxide tension, and starvation, all of which depress the rate of metabolism of animals, also lower the positive rheotactic response of isopods. High oxygen, caffeine, and a gradual increase in temperature have the opposite effect.

A desire to obtain more exact information regarding this correlation of metabolic rate and the rheotactic reaction of isopods led to an attempt to measure the rate of metabolism by means of the survival-time in potassium cyanide. This method was worked out by Professor Child ('13) for planarians, and is essentially as follows:

In a relatively strong solution of potassium cyanide (0.001 mol. or thereabouts) the animals having the highest rate of metabolism die first, or in other words, they have the shortest survival-time. Those having the lowest rate die last and hence have the highest rate of metabolism. Child called this the 'direct method' of

¹ The experiments upon which this paper is based were performed in the Laboratory of Plant Physiology at the University of Illinois and in the Marine Biological Laboratory, Woods Hole, Mass. I am indebted to Dr. C. F. Hottes and Prof. F. R. Lillie for furnishing facilities for carrying on the experiments. I am also indebted to Prof. C. M. Child and to Dr. Shiro Tashiro for many helpful suggestions, and to Marjorie Hill Allee for assistance with certain experiments and for aid in tabulating results.

measuring the resistance of the animal. The effect is due to the action of the cyanide which (Geppert '99) decreases the ability of the tissues to take up oxygen. Child ('13 a) suggests that the effect of the potassium cyanide solution depends upon the number of chemical bonds in the organism which are opened in a given length of time. This means that the higher the rate of reaction, the greater is the opportunity of the cyanide to produce its effect. In a much weaker solution (0.00001 mol.) acclimatization occurs and the animals with the higher rate of reaction live longer than those with a lower rate. This is called the 'indirect method.'

Although Professor Child found this method held for planarians it did not necessarily follow that the chitinous-covered, gill-breathing isopods would be affected in the same manner. My problem then was, first, to find whether or not the cyanide method would hold for isopods and then whether or not there was a relation between the rheotactic reaction and the resistance to potassium cyanide.

THE STOCK

Three groups of *Asellus communis* were tried in these experiments:

I. The offspring of a stock that was originally collected in August, 1911, from County Line Creek, north of Glencoe, Illinois (Shelford '11 a, maps) and which had been in well-aerated laboratory aquaria since (Allee '13). These isopods were half-grown when used.

II. A large number of breeding isopods collected in the above stream April 13, 1913, and kept under the same conditions as the first group. Both adults and young were tested.

III. A series of collections of pond isopods, also *A. communis* but mainly juvenile, from small fresh-water ponds near Woods Hole, Massachusetts. These came from silt- and debris-bottomed ponds, low in oxygen. They were kept under similar conditions in the laboratory.

These groups will be referred to in the body of the paper as Stocks I, II and III respectively. The mortality rate of all three groups was low while under observation. During the course of

these experiments individual tests were made of the reactions of 257 isopods of the first two groups, and of 195 of the third lot, making a total of 452 isopods. Moreover, these experiments consistently support results of the preceding four years experimental study on this subject.

METHODS

The isopods were tested for their rheotactic reaction in a circular pan the bottom of which was covered with wax. The responses of an individual isopod for ten successive one-minute reaction periods were taken as a fair indication of the rheotactic tendencies of the animal. The approximate distance covered during each reaction was recorded and will be found in a standardized form in the tables under the head of efficiency in the current.²

Immediately following the rheotaxis test the animals were placed in potassium cyanide solution in Erlenmeyer flasks of 150 to 2000 cc. capacity. The size of the flask depended upon the amount of oxygen to which the isopod was accustomed and upon the strength of cyanide used. In all instances, had the cyanide been omitted the isopods would have shown no ill effects of confinement for several days. A 0.1 molecular solution of potassium cyanide was made up in distilled water. From this, solutions of the desired strength were made in water similar to that in which the isopods had been kept. During the survival periods in the flasks the temperature was the same as in the stock aquarium.

Exact determination of the death point gave considerable difficulty. It was found that close examination with a lens for

² The following arbitrary standard of efficiency in the current was adopted in the earlier experiments and is used in those reported here. This represents numerically the distance covered by an isopod in a minute's reaction period:

- 0 no reaction
- 1 slight movement
- 2 any response between 1 and 3
- 3 progress one-third around the pan positive (approximately 27 cm.) or two-thirds, negative (54 cm.)
- 4 progress two-thirds around the pan positive or one and one-third negative
- 5 progress once around the pan positive or twice around, negative
- 6 any distance over 5

For further details see Allee '13.

movement of the gills or for slight twitching of the anterior legs gave the most satisfactory results. About the time of death the pleopods which act as gill covers move out until they stand almost at right angles with the body and the gills become more or less swollen. As a check upon the supposed death-time, observations were carried on for an interval after the animals were apparently dead. In doubtful cases or with very small isopods, the animals were removed from the flask with a large pipette and examined in a watch-glass under a dissecting microscope. This was never done until movement had almost ceased and the stimulation did not appreciably affect results.

TESTING THE CYANIDE RESISTANCE METHOD

If the resistance to potassium cyanide is a measure of the metabolic condition of isopods, then, other things being equal, the higher the temperature the greater the activity in the cyanide, and the younger the animal the sooner it should die. Also the greater the output of carbon dioxide, the shorter should be the survival time. That all these requirements are fulfilled by the cyanide method with isopods is shown by the tests recorded in table 1.

Raising the temperature of isopods during the survival period gave a very marked decrease in the survival time. In making the tests recorded in table 1, the isopods were placed in 0.001 molecular potassium cyanide solution at the same temperature as the water in which they were tested for their rheotactic reaction (18–21°C.). As a control, other isopods as nearly similar as could be selected were placed in an equal amount of water. Both lots were then gradually heated to 30 to 32°. In all cases the isopods in cyanide died in from thirty-five minutes to an hour and forty minutes, a length of time much below the average survival-time of similar isopods in cyanide of the usual temperature. Furthermore, in all instances the isopods in water survived the heating process without being visibly affected by it.

In other experiments an attempt was made to keep isopods at about 25°C. (5 to 8° above the temperature of the stock at that

time) for a week before killing them in cyanide at the same temperature. Under these conditions there was a shortened survival-time but the variations in temperature were too great for the results to be anything but indicative.

In the work with lowered temperature the isopods had been in a temperature of 17 to 19° and were placed in an ice-chest with the temperature at 8 to 14°. After a week under these conditions they were killed in potassium cyanide, 0.0002 molecular solution, at the same low temperature. The isopods at the usual temperature (table 1) showed a higher rate of positive rheotaxis and of efficiency in the current than did those from the lower temperatures and hence would be expected to give a shorter survival-time regardless of the temperature change, but the difference is entirely too great to be altogether accounted for in this way.

It is to be expected that the chemical activity of the cyanide at low temperature would be less than at a higher temperature, but it is highly improbable that the great differences in survival-time at the different temperatures could be due to this cause

TABLE 1

Showing the effect of different factors upon the survival in potassium cyanide with a view to testing whether the resistance to cyanide bears any relation to the metabolic condition of isopods

STOCK	EXPERIMENTAL CONDITIONS	MOLECULAR STRENGTH OF KCN SOLUTION	NUMBER ISOPODS USED	AVERAGE RHEOTACTIC REACTION IN PERCENTAGE OF TOTAL TRIALS	AVERAGE EFFICIENCY	AVERAGE LENGTH IN MM.	AVERAGE SURVIVAL-TIME IN HOURS AND MINUTES	RANGE OF TEMPERATURE
				+	-			
				8	0			
III	Heightened temperature	0.001	10 42	51	7	2.2	5.0	1.03 30-32
III	Usual temperature.....	0.001	55 50	37	10	2.2	6.0	5.14 18-21
II	Lowered temperature....	0.0002	9 46	35	19	2.2	12.4	30.17 8-14
I-II	Usual temperature.....	0.0002	72 61	28	11	2.5	10.7	5.59 17-21
II	Stimulation by shaking.	0.0002	6				15.5	2.19 17-21
II	Not stimulated: control	0.0002	9				14.8	5.12 17-21
I-II	Not stimulated: average	0.0002	72 61	28	11	2.5	10.7	5.59 17-21
III	Stimulated by shaking..	0.001	9 31	32	32	1.6	4.4	2.39 18-19
III	Not stimulated: average	0.001	55 50	37	10	2.2	6.0	5.14 18-21
I-II	12 or more mm. long....	0.0002	20 59	27	14	2.3	14.2	6.25 17-21
I-II	8 or less mm. long.....	0.0002	20 60	23	17	2.4	7.4	5.04 17-21

alone. Moreover, the effect of temperature upon metabolism will adequately explain the difference found to exist. Therefore the results of measuring the survival-time of isopods at low and at high temperatures, while not a complete demonstration, strongly indicate that the resistance to potassium cyanide is a measure of metabolic rate in isopods.

In the case of stimulation of the isopods by shaking them while exposed to the cyanide, there is a marked decrease in the survival-time. This is what would be expected if the resistance to potassium cyanide is a measure of metabolic activity. In these experiments the isopods were shaken every few minutes as long as this stimulated them to activity. If objection be made that shortening of the survival-time under these conditions is a fatigue response, it is only necessary to point out that fatigued animals would have a lower rate of metabolism and hence a longer survival time than unstimulated animals. Consequently, if fatigue enters as a factor, it tends to lengthen, not shorten, the survival-time. But it is improbable that fatigue is a factor in the survival-time of these stimulated isopods since they were usually lying on their backs unable to respond to stimulation before either the pond or stream mores would have shown a change in their rheotactic reaction under continuous stimulation.

The figures shown in table 1 regarding the effect of size upon the survival show that the larger the animal the longer is the survival-time in potassium cyanide. These results are clear-cut in all respects save one. The rheotaxis both in percentage of responses and in efficiency of the reaction is practically identical, while the size differences and the survival-time differences are marked. But almost all the larger ones are male and most of the smaller ones are female and most of the records were taken during the breeding season. The females of the same age are usually smaller than the males, but not so much smaller as is here indicated. From general appearance these females were younger than the larger ones that were apparently the same age as the males.

An examination of the records of 58 males and 28 females from Stock III, mainly out of the breeding period, in which the size and rheotactic responses averaged approximately the same,

showed no definite difference in the survival-time of the two sexes. If anything, the females had the longer survival-time. This would tend to rule out the question of sex in the cases above noted but leaves that of the effect of the breeding season unanswered. The results may fairly be interpreted as showing a difference in survival-time due mainly to difference in age and perhaps partially to a differential action of breeding responses on males and females.

Very small isopods (1.5–1.75 mm. long) have a much reduced survival-time. Ten of this size lived only three hours and forty minutes in 0.0002 molecular potassium cyanide solution, while the mean for 72 adults averaging 10.7 mm. long, was five hours and fifty-nine minutes. This marked difference may have been partially due to the more tender external covering of the smaller isopods which allowed more direct action of the cyanide, but since the facts now at hand indicate that recently molted isopods live a longer, not a shorter, time than their mates, this does not seem sufficient ground to account for so great difference.

Finally, the most crucial evidence that resistance to cyanide does measure metabolism in isopods, is to be found in a comparison between the carbon dioxide output and the survival-time. A number of determinations of this character were made jointly with Dr. Shiro Tashiro at Woods Hole during the past summer (cf. Tashiro '13 p. 141). The results of these experiments will be published in detail elsewhere (Allee and Tashiro '14). Suffice it to say that in the twelve trials made, with one easily-explained exception, the resistance to potassium cyanide was inversely proportional to the rate of carbon dioxide production. As the experiments were run, this is the most delicate and accurate test and it is the more interesting in that the results with this method were quantitative.

In summing up the inquiry as to whether the resistance to relatively strong solutions of cyanide does measure the metabolic conditions of isopods, it is found that the survival-time is decreased by raising the temperature and by stimulation by shaking; that it is lengthened by decreasing the temperature; that larger (older) isopods have a longer survival-time than small (young) ones; and that the carbon dioxide production is inversely proportional to

the resistance to cyanide. From this evidence it seems safe to conclude that the cyanide resistance method does measure the metabolic state of isopods.

RELATION BETWEEN RHEOTAXIS AND SURVIVAL-TIME IN POTASSIUM CYANIDE

The direct method

From preceding work on this subject (Allee '12, '13) one would expect that the isopods giving the most highly positive rheotactic reaction, other things being equal, would die sooner in potassium cyanide than any others; that those giving a highly negative response would die next; that those with a high percentage of indefinite reactions would have a still longer survival-time; and that isopods that failed to respond to the current at all would live the longest. The results of experimental tests on the subject are listed in table 2.

The results from 150 individuals exhibited in table 2, in which a high percentage of positive reactions are contrasted with a low

TABLE 2

Showing the relation between survival-time in potassium cyanide and the different rheotactic responses. Temperature 17 to 21° C.

STOCK	RHEOTACTIC RESPONSES COMPARED	MOLECULAR STRENGTH OF KCN SOLUTION	NUMBER ISOPODS USED	AVERAGE RHEOTACTIC REACTION IN PERCENTAGE OF TOTAL TRIALS				AVERAGE EFFICIENCY	AVERAGE LENGTH IN MM.	AVERAGE SURVIVAL-TIME IN HOURS AND MINUTES
				+	-	8	0			
I-II	60 per cent or more +	0.0002	41	88	7	5		2.64	10.2	5:24
	60 per cent or more -	0.0002	19	13	83	5		2.63	10.2	6:22
	40 per cent or less +	0.0002	25	16	63	21		2.54	10.4	6:39
III	60 per cent or more +	0.0002	26	83	9	8		2.34	7.4	21:16
	60 per cent or more -	0.0002	17	6	88	6		2.21	6.8	22:33
	40 per cent or less +	0.0002	23	15	71	14		2.12	6.8	23:31
III	60 per cent or more +	0.001	26	83	12	3		2.33	6.2	4:34
	60 per cent or more -	0.001	16	5	91	4		2.24	5.9	5:15
	40 per cent or less +	0.001	24	9	68	17	6	1.98	6.0	5:34
	60 per cent or more 0	0.001	14	0	0	17	83	0.16	6.4	3:00

percentage, show in each case that the lower rate of positive rheotaxis accompanies a relatively long survival-time in the cyanide. That is, the isopods giving a low positive rheotactic reaction have on the average a slower rate of metabolism than do animals giving a higher percentage of positive responses. We are dealing here with average results only; individual results will be considered later (p. 410).

The position in the metabolic gradient of isopods that give a high percentage of negative reactions has been discussed in an earlier paper (Allee '13). From the evidence then at hand it was decided that under these conditions isopods occupied a place midway between that which accompanies a high degree of positiveness and a high degree of indefiniteness. That this conclusion is true is indicated by the results given in table 2, where averaged responses of 52 individuals from three sets of experiments have a survival-time intermediate between that given with a high percentage and that with a low percentage of positive responses.

In all the individuals tested in these experiments the negative rather than the indefinite response increased as the positive response lessened. For this reason there were too few instances where the isopods gave a majority of indefinite reactions to make their inclusion in the table of any value. However, the position upon the metabolic gradient of animals giving a high percentage of indefinite responses is firmly established by the earlier experiments (Allee '12, '13). These clearly show that the indefinite reaction accompanies a rate of metabolism lower than either of the other responses.

The survival-time of animals showing a high percentage of inaction in the current varies greatly. At times the survival-time is much longer than when movement occurs but on the average it is shorter than that of highly positive isopods. This is not surprising if one remembers that there is no rheotactic method for measuring the metabolic activity of the isopods that give no reaction to the current. They may be just lower than the lowest rate that would result in an indefinite reaction, or they may not be strong enough to turn over on their feet after the current has turned them on their backs. It is the first type that gives the

long survival-period while the very weak ones die soon after being placed in the cyanide. In this regard the cyanide method would fail to measure the metabolic rate. This may be due to there being just enough oxidation carried on to keep the isopod alive, so that when the cyanide decreases this ever so little death speedily results.

In another paper (Allee '13) it was pointed out that the efficiency of reaction in the water current varied more or less closely with the percentage of positive responses. That this is also an expression of the metabolic condition of the isopod is shown by the data presented in table 3.

TABLE 3

Showing the relation between the survival-time in potassium cyanide and the efficiency of the response to the current of water. Temperature 17 to 21° C.

STOCK	EFFICIENCY OF RESPONSES COMPARED	MOLECULAR STRENGTH OF KCN SOLUTION	NUMBER ISOPODS USED				AVERAGE RHEO- TACTIC REACTION IN PERCENTAGE OF TOTAL TRIALS	AVERAGE EFFICIENCY	AVERAGE LENGTH IN MM.	AVERAGE SURVIVAL- TIME IN HOURS AND MINUTES
			+	-	s	0				
I-II	2.8 or more	0.0002	27	71	26	3	3.1	9.6	6.21	
	2.0 or less	0.0002	13	43	18	36	1.7	11.4	7.14	
III	2.6 or more	0.0002	5	60	40		2.9	8.9	14.46	
	2.0 or less	0.0002	10	32	21	39	1.7	6.3	19.49	
	2.6 or more	0.001	10	63	34	3	2.8	7.0	4.59	
	2.0 or less	0.001	19	35	38	22	1.78	5.9	5.11	

The results exhibited in table 3 are too few to be more than indicative; however, since they agree with all the other observations I think they approximate the truth of the situation. This is that while high efficiency in the current usually accompanies a larger percentage of positive responses and a higher rate of metabolism than low efficiency, yet considered alone the differences are neither so marked nor so sure as when the sign of the rheotactic reaction is made the index of the metabolic condition.

One pertinent comparison remains in the relation between survival-time and rheotaxis. What are the rheotactic reactions

which accompany relatively long and relatively short resistance to potassium cyanide? A synopsis of these results is presented in table 4.

Upon making this comparison one is struck, first by the relative closeness of the rheotactic responses given under the two conditions and second by the fact that the longer survival-times have always the lower percentage of positive responses. One of the factors causing the relatively low percentage of positive rheo-

TABLE 4

Showing the relation between long and short survival-time and rheotaxis. Temperature 17 to 21° C.

STOCK	SURVIVAL-TIMES COMPARED	NUMBER ISOPODS USED	AVERAGE SURVIVAL-TIME IN HOURS AND MINUTES				AVERAGE RHEOTACTIC REACTION IN PERCENTAGE OF TOTAL TRIALS		AVERAGE EFFICIENCY	AVERAGE LENGTH IN MM.	MOLECULAR STRENGTH OF KCN SOLUTION
			+	-	8	0					
I-II	5 hours or less...	29	4:03	65	25	10	2.5	10.5	0.0002		
	7 hours or more...	20	9:16	50	32	18	2.5	11.1	0.0002		
	18 hours or more...	21	9:09	56	32	10	22.3	7.3	0.0002		
III	28 hours or more.....	18	39:10	47	41	9	32.1	6.5	0.0002		
	4 hours 15 minutes or less..	20	3:01	51	38	8	32.1	6.1	0.001		
	6 hours 15 minutes or more	16	8:58	34	43	18	52.1	6.1	0.001		

tactic reactions with the shorter survival-periods is that isopods sometimes give a high degree of negativeness with a relatively short survival-time (cf. Allee '13), although on the average such isopods live longer in the cyanide than do highly positive ones (see also p. 405).

The indirect method

Only isopods from Stock II were tested by this method. These were placed in 0.00001 molecular potassium cyanide solution in two-liter flasks immediately after having been tested for rheotaxis. Three easily distinguished individuals were placed in each flask. In order to secure more even temperature the flasks were placed in running water. The temperature averaged 18° C., but

varied $\pm 4^\circ$. The isopods were examined each morning and night. As soon as one died it was removed with a long pipette. In general the method is, I believe, a trifle more reliable than the direct method since the exact determination of the death point is of less significance. However, since the method is so much slower it was used only to check the results obtained with the direct method. The findings are summarized in table 5. They support, as far as is possible with a limited number of cases, the findings with the direct method.

TABLE 5

Showing the relation between survival-time in 0.0001 molecular potassium cyanide solution and the rheotactic reaction in Stock II. Temperature range 14 to 22° C.; average 18°

RHEOTACTIC RESPONSES COMPARED	NUMBER ISOPODS USED	AVERAGE RHEOTACTIC REACTION IN PERCENTAGE OF TOTAL TRIALS				AVERAGE EFFICIENCY	AVERAGE LENGTH IN M.M.	AVERAGE SURVIVAL-TIME IN DAYS
		+	-	∞	0			
60 per cent or more + . . .	19	86	5	9		2.7	9.6	5.7
40 per cent or less + . . .	12	16	61	20	3	2.4	10.0	2.5

DISCUSSION

It was shown in an earlier paper (Allee '12) that isopods of the pond mores are less positive in their rheotactic reaction than those of the stream mores. This is again illustrated by these studies and light is thrown on the metabolic condition of the isopods giving this difference in response. Thus the rheotactic reactions of 72 isopods of the stream mores (Stocks I and II), taken partially during the lowered responses of the breeding season, averaged 61 per cent positive, 28 per cent negative, and 11 per cent indefinite, with an average efficiency in the current of 2.5. These gave a mean survival-time in 0.0002 molecular potassium cyanide of five hours and fifty-nine minutes. ♦

Of the pond mores 51 were killed in 0.0002 molecular cyanide solution with an average survival-time of twenty-three hours and

ten minutes. These pond isopods had given an average rheotactic response of 51 per cent positive, 37 per cent negative, 10 per cent indefinite and 2 per cent no reaction. Their average efficiency in the current was 2.2. Another lot of 55 pond isopods which gave a rheotactic average similar to the above, were killed off from normal conditions in 0.001 molecular potassium cyanide; these gave a mean resistance of five hours and fourteen minutes, just forty-five minutes less than that given by the stream isopods to a cyanide solution one-fifth as strong.

All this means that the stream isopods, the ones that as a rule are more highly positive to and more efficient in their reactions in a water current than pond isopods, have a shorter survival-time in equimolecular solutions of potassium cyanide and therefore have a higher rate of metabolism than do the pond isopods.

If only the highly positive isopods of the two mores are chosen for a comparison of their resistance to potassium cyanide, another situation is revealed. The individuals of the two groups selected on the basis of the similarity of their rheotactic response show a great difference in their survival-time in equimolecular solutions of the cyanide and hence in their metabolic rate. In table 2 (p. 404) the stream isopods with a rheotactic reaction 60 per cent or more positive averaged 88 per cent positive and gave a mean survival-time in 0.0002 molecular potassium cyanide of five hours and twenty-four minutes. Isopods from the pond mores selected on the same basis averaged 83 per cent positive and gave a survival-time of twenty-one hours and sixteen minutes, in the same strength of cyanide solution. Here is a great difference in the metabolic activity of the two groups of isopods but a close similarity in their rheotactic reaction. It can also be seen in table 2 that the pond isopods that give a low positive response have a longer survival-time in potassium cyanide than those that are more highly positive. This must mean that the rate of metabolism which is accompanied by a high degree of positiveness is relatively high when compared with the mean metabolic rate given under the conditions to which the isopods are acclimated, but that it may not be an absolutely high rate when compared

with a fixed standard. This topic will immediately be discussed more fully in dealing with the reactions of individual isopods.

Thus far I have been dealing with averaged results. For generalizations these are necessary and in order to save space the individual records have not been given. How far does the work with individuals support the averaged results? Before taking up this point it is necessary to point out that each individual has in all probability a rate of metabolic processes slightly different from that of any other isopod and further, that it is not a fixed standard rate of metabolism that finds its expression in a high rate of positiveness but rather a relative rate. That is, when the metabolism of an isopod is rapid for that individual it tends to go positive to the water current, when less rapid, negative, when still less rapid, indefinite, and when least rapid no reaction at all is given. But a rate of metabolism that is rapid for one isopod may be slow for another, and intermediate for a third. Now all three would show the same survival-time in potassium cyanide and yet their rheotactic reaction would be highly different. Thus for example, isopods 96 and 101 from Stock III had a survival-time of three hours and forty-five minutes in 0.001 molecular cyanide solution. The former gave 50 per cent positive, the latter 100 per cent positive reactions. Isopod No. 97 was killed in the same solution with the other two with a survival-time of three hours and fifteen minutes, yet its rheotactic reaction was only 60 per cent positive. Since it is obviously impossible to test the survival-time of an isopod in its varying rates of metabolism, one is forced to do the next best thing, that is, take average results.

There is still another complication in this regard. An individual isopod may give identical rheotactic reactions when its rate of metabolism varies greatly. Thus an isopod kept at 20°C. comes to have a normal mean metabolic rate, also a normal mean rheotactic response. When the metabolic rates goes above this normal mean the isopod tends to become more positive, when below, less positive to water currents. Put the same isopod at 5°C. The metabolic rate is depressed and the positiveness also decreases. But in time the isopod becomes acclimated; a new metabolic mean is established and the rheotactic reaction goes up

to about its old average and plays up and down as the metabolic rate varies about its new mean. So the rheotactic reaction is an expression, not of the absolute metabolic rate of the animal, but of the relative metabolic conditions under which the isopod is acclimated for the time being. Further experimental evidence on this point will be published elsewhere in conjunction with Dr. Shiro Tashiro.

But even with this relative scale of metabolism determining the rheotactic reaction of the isopod in place of an absolute scale, the fact remains that individuals having a short survival-time usually have a high rate of positiveness to a water current. This was determined for individuals as follows: An assistant read me the rheotactic reaction of isopods in groups of two from the same stock, giving individuals that had at least 10 per cent of difference in their positive rheotactic response. Basing judgment on the rheotactic reaction alone I was able to predict the relative survival-time in 106 out of the 160 cases tried. This shows that the rheotactic reaction in 66 per cent of the cases was a correct indicator of the absolute metabolic conditions of the isopods compared. Isopods with a highly negative reaction gave the most difficulty, while it was impossible to predict the survival-time of isopods that did not move at all in the current. The explanation of this difficulty has already been given (pp. 405-406).

Jennings ('06) clearly outlined the problems in this particular field of animal behavior when he said, "We are compelled to assume the existence of changing physiological states. This assumption besides being logically necessary, is of course supported by much positive evidence drawn from diverse fields, and there is reason to believe that in time we shall be able to study these states directly."

With the application of the cyanide method to problems of animal behavior this prophecy is a step nearer its final fulfilment and it is now possible to demonstrate directly as regards the rheotactic reaction of isopods that high positiveness is the expression of a relatively high rate of metabolism, and low positiveness, of a low metabolic rate.

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STUDIES ON THE DYNAMICS OF MORPHOGENESIS AND INHERITANCE IN EXPERIMENTAL REPRODUCTION

VII. THE STIMULATION OF PIECES BY SECTION IN PLANARIA DOROTOCEPHALA

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FOUR FIGURES

I. METHODS

By means of the direct susceptibility method it is possible to follow the changes in rate of metabolism resulting from section in pieces. This method, which has been described in detail elsewhere (Child '13 a), consists in determining the susceptibility of individuals or pieces to concentrations of KCN, alcohol, and so forth, which kill within a few hours. In such concentrations the higher the general rate of metabolism the greater the susceptibility and the shorter the life of the animal or piece in the solution. In this way the susceptibility serves as a means of distinguishing and comparing the general rate of metabolism in different individuals, regions, or pieces. Since the changes in susceptibility following section depend upon size of piece and region of the body from which it is taken it has been found most satisfactory to determine the susceptibility of series of pieces, representing both different fractions of the body length and different regions of the body, at various intervals after section. Of course a different series of pieces must be used for each susceptibility determination but by standardizing the material used and the external conditions a remarkable uniformity of results is possible.

The method of experiment is as follows: The bodies, exclusive of the heads, of ten worms of equal length and similar physiologi-

cal condition and always from the same stock, are cut into fourths, sixths, eighths, or twelfths; each lot of ten pieces representing approximately the same region of the body is kept together and isolated from other lots and its susceptibility tested at the desired time after section.

In all the susceptibility determinations of pieces presented below animals from the same stock were used and great care was exercised in selecting those of equal size, as experience has shown that in a particular stock size is the best criterion of physiological condition, and also in cutting the pieces so that they should represent as nearly as possible the same regions of the bodies of different individuals. It is of course impossible to avoid some differences in size and level of pieces but the results will show how unimportant these differences are. In all susceptibility determinations KCN 0.001 *m* was used in filtered water from the same source and at the same temperature and the same quantity of KCN solution was used in each case.

In this manner the susceptibility of series of ten each of fourths, sixths, and eighths, of wellfed animals 18 mm. in length has been determined, immediately, twelve, twenty-four, forty-eight, seventy-two, ninety-six and one hundred and twenty hours after section, and for some series of pieces three and six hours after section also. For twelfths the susceptibility has been determined, immediately, twenty-four and forty-eight hours after section, but it is impossible to avoid considerable relative differences in size in pieces as small as these and such differences complicate the results. With pieces smaller than twelfths no general susceptibility determinations have been attempted.

"Immediately after section" in these series means that as soon as the cutting of the series of pieces was completed they were placed in KCN. Since the preparation of the series requires from ten minutes to an hour according to the size and number of pieces the first pieces cut remain in water for some time before being placed in KCN. It was ascertained, however, that this made no difference in the results since the susceptibility does not change appreciably during several hours after section. In nearly

all cases the determination for each size and each time has been made repeatedly so that conclusions do not rest on single series and besides this the results of these experiments are amply confirmed by large numbers of others in which only certain regions of the body instead of the whole were used in susceptibility determinations in relation to size and region.

In the susceptibility determinations, as stated the whole body except the head was cut into fourths, sixths, etc., and the susceptibility of all pieces was determined and recorded, but in the following tables only the pieces from the first zoöid (see Child '11 e) are included. The pieces of the posterior zoöids show nothing fundamentally different from those of the first zoöids and double the length of the tables. Only a sufficient number of susceptibility records are given to show the general course of the changes in susceptibility. The tables of these records given below are supplemented and confirmed by many other similar series in my notes.

The tables are in the form of the susceptibility tables in an earlier paper (Child '13 a). The column headed "length of time" gives in hours and minutes the length of time that the animals or pieces have been in the solution at each observation. The column headed "lots" gives the serial numbers for each time of the different lots of ten each, beginning with the most anterior pieces as Lot 1. The lot-numbers are the same as the numbers of the pieces in the corresponding figures.

The five columns headed "I to V" under "stages of disintegration" distinguish more or less arbitrarily five stages in the visible changes which the animals undergo in dying. These five stages are briefly as follows:

I. Still intact, showing no appreciable disintegration. Such animals or pieces are always alive and moving about.

II. In whole animals, from the first traces of disintegration to the beginning of disintegration along the margins of the body, which usually follows disintegration of the head. In pieces from the beginning of disintegration at one or both ends to beginning of disintegration along the lateral margins. Considerable motor activity may still be present.

III. In both whole animals and pieces disintegrations of lateral margins of body until it is completed and swelling of body begins. Movement may still occur to some extent in the intact parts.

IV. Swelling of the body to complete loss of epithelium and loss of shape. Motor activity has ceased.

V. All further changes after swelling and loss of shape are completed.

The recognition of these different stages makes it possible to distinguish more clearly slight differences in susceptibility which would otherwise not be evident.

The numbers in each of the five columns for each time and each lot are the actual numbers of animals or pieces of that lot which are in that stage at that time. Lots of ten each were used and condition was recorded every half-hour in all cases.

From these tables it is possible to determine at a glance the regional differences in susceptibility and by comparison of the tables for pieces of different size the effect of size and region upon the changes in susceptibility following section appears and comparison of the susceptibilities of pieces of the same size at different times shows the course of the susceptibility changes.

II. STIMULATION

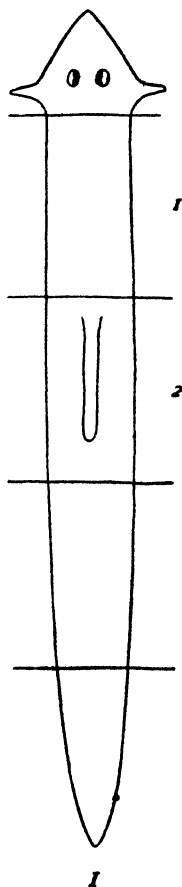
1. The susceptibility of whole animals

In order to show how the susceptibility of the pieces changes following section it is necessary to determine the susceptibility of whole animals as a basis for comparison. Table 1 gives the susceptibility of a lot of ten worms 18 mm. in length, that is, of the same size and physiological condition as the animals used for the preparation of pieces.

2. The one-fourth pieces

The regions of the body represented are indicated in figure 1. Pieces 1 and 2 represent the body of the first zoöid and only the records of these pieces are included in the tables.

Comparison of table 2, the one-fourth pieces, immediately after section, with table 1, the whole worms, shows that the susceptibility of the pieces is somewhat greater than that of the whole animals. Susceptibility determinations twelve and twenty-



four hours after section show a lower susceptibility than table 2 but no great change occurs during this period. Table 3, forty-eight hours after section, shows a susceptibility lower than that of whole animals.

TABLE 1
Susceptibility of ten worms 18 mm. in length to KCN 0.001 m

LENGTH OF TIME IN KCN	STAGES OF DISINTEGRATION				
	I	II	III	IV	V
4.00	4	6			
4.30	4	5	1		
5.00		7	3		
5.30		3	7		
6.00			10		
6.30			10		
7.00			7	3	
7.30			4	4	2
8.00				4	6
8.30				2	8
9.00					10

TABLE 2
Susceptibility to KCN 0.001 m of one-fourth pieces immediately after section

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
3.30	1	7	3			
	2	9	1			
4.00	1		9	1		
	2	2	8			
4.30	1		6	3	1	
	2		7	3		
5.00	1			7	3	
	2		2	7	1	
5.30	1			7	3	
	2			9	1	
6.00	1			2	7	1
	2			6	4	
6.30	1				7	3
	2				9	1
7.00	1				4	6
	2				5	5
7.30	1					-10
8.00	2				1	9
	2					-10

TABLE 3

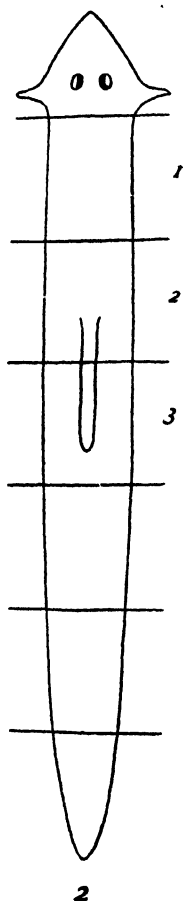
Susceptibility to KCN 0.001 m of one-fourth pieces forty-eight hours after section

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
5 30	1	9	1			
	2	10				
6 00	1	4	6			
	2	8	2			
6 30	1	1	9			
	2	8	2			
7 00	1	1	5	2	2	
	2	6	2	2		
7 30	1	1		5	2	2
	2	2	3	3	2	
8 00	1		1	2	2	5
	2	2	3	3	1	1
8 30	1			1	2	7
	2		2	3	3	2
9 00	1			1	1	8
	2		2	1	3	4
9 30	1				1	9
	2			2	3	5
10 00	1					10
10 30	2				2	8
11 00	2					10

TABLE 4

Susceptibility to KCN 0.001 m of one-fourth pieces one hundred and twenty hours after section

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
3 00	1	7	3			
	2	4	6			
3 30	1		9	1		
	2		4	6		
4 00	1		5	4		1
	2		4	4	2	
4 30	1		4	4		2
	2		4	3	3	
5 00	1		4	1	3	2
	2			4	4	2
5 30	1		2	2	1	5
	2				5	5
6 00	1			2	1	7
	2					10
6 30	1				3	7
7 00	1					10



From forty-eight hours on, the susceptibility begins again to increase as the pieces undergo reconstitution. At seventy-two hours it is somewhat greater, at ninety-six hours still greater and table 4 for one hundred and twenty hours shows that it has now again become greater than that of the whole worms (cf. table 1).

As regards the susceptibility of the first and second fourths, tables 2 to 4 show that the first fourth is at all times more susceptible than the second, that is, the original axial gradient (Child 12 a, 13 b, 13 c) appears in the difference in susceptibility between the first and second fourths.

3. The one-sixth pieces

Figure 2 shows the region of body included in each of the one-sixth pieces. In table 5 to 8 only records of the pieces 1, 2 and 3 which represent the body of the first zoöid are included.

Comparison of tables 5 to 8 with each other and with table 1 shows that the susceptibility of the one-sixth pieces immediately after section (table 5) is greater, but after twelve hours (table 6) is less than that of whole worms.

TABLE 5

Susceptibility to KCN 0.001 m of one-sixth pieces immediately after section

LENGTH OF TIME IN KCN	LOT#	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
3.30	1	10				
	2	7	3			
	3	7	3			
4.00	1	7	3			
	2	4	6			
	3	4	6			
4.30	1	2	8			
	2	1	6	3		
	3	2	6	2		
5.00	1	2	6	1	1	
	2		2	6	2	
	3		4	5	1	
5.30	1	2		7	1	
	2			6	3	1
	3		1	7	1	1
6.00	1		2	4	1	3
	2			2	6	2
	3			3	2	5
6.30	1		2	1	2	5
	2				5	5
	3			1	3	6
7.00	1			2	1	7
	2				4	6
	3				1	9
7.30	1				1	9
	2					10
	3					10
8.00	1					10

Susceptibility determinations twenty-four and forty-eight hours (table 7) after section show a slight further decrease in susceptibility. From this time on the susceptibility begins to increase as reconstitution proceeds. At seventy-two hours it has increased slightly, at ninety-six still more and at one hundred and twenty hours it is once more higher than that of the whole worm. As reconstitution goes on it continues to increase.

These sixths show another interesting feature. Comparison of Lots 1, 2 and 3 in table 5 shows that 2 and 3 are considerably

TABLE 6

Susceptibility to KCN 0.001 m of one-sixth pieces twelve hours after section

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
5 00	1	9	1			
	2	10				
	3	8	2			
5 30	1	7	3			
	2	10				
	3	7	2	1		
6 00	1	5	4	1		
	2	8	2			
	3	6	2	2		
6 30	1	4	3	2	1	
	2	5	3	2		
	3	3	1	3	2	1
7 00	1		4	3	2	1
	2	5		3	2	
	3	1	2		4	3
7 30	1			4	1	5
	2	4	1	1	2	2
	3		2	1	1	6
8 00	1			1		9
	2	1	2	2		5
	3		1		2	7
8 30	1				1	9
	2		1	2	1	6
	3			1		9
9 00	1					10
	2			2		8
	3					10
9 30	2				2	8
10 00	2					10

more susceptible than 1; in other words, the susceptibility of posterior is greater than of anterior pieces. This change constitutes, so far as the different pieces are concerned, a reversal of the axial gradient of the first zoöid of uninjured worms. It is a characteristic feature not only of sixths but, as will appear below, of all shorter pieces and is due to the fact that posterior pieces are more stimulated by the act of section than anterior.

After twelve hours (table 6) the effect of this stimulation has disappeared in Lot 2 so that its susceptibility is lower than that

TABLE 7

Susceptibility to KCN 0.001 m. of one-sixth pieces forty-eight hours after section

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
6.00	1	9	1			
	2	10				
	3	10				
6.30	1	7	3			
	2	10				
	3	10				
7.00	1	4	4	2		
	2	10				
	3	10				
7.30	1	2	2	3	3	
	2	9		1		
	3	9	1			
8.00	1		2	1	2	5
	2	5	3	1	1	
	3	4	3	2	1	
8.30	1			2		8
	2	1	6	1	1	1
	3	2	4	1	2	1
9.00	1				2	8
	2	1	3	1	5	
	3	2	1	1	3	3
9.30	1					10
	2	1			6	3
	3		1		3	6
10.00	2	1				9
	3				1	9
10.30	2					10
	3					10

of Lot 1 while Lot 3 has not yet fully recovered and its susceptibility is about the same as that of Lot 1.

After twenty-four hours further recovery has occurred and after forty-eight hours (table 7) the susceptibility of Lot 2 is lower than that of Lot 1 and that of Lot 3 slightly lower than that of Lot 2, that is, the original axial gradient of the whole worm has now reappeared in the relative susceptibilities of the pieces.

As reconstitution proceeds, the susceptibility of all the pieces rises and since the more posterior regions of the first zoöid undergo more reorganization than the anterior regions in giving rise to a new individual, the susceptibility of the posterior pieces rises

TABLE 8

Susceptibility to KCN 0.001 m. of one-sixth pieces one hundred and twenty hours after section

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
3.00	1	4	6			
	2	6	4			
	3	9	1			
3.30	1		10			
	2		10			
	3	3	7			
4.00	1		9	1		
	2		10			
	3	3	7			
4.30	1		4	6		
	2		7	3		
	3		7	3		
5.00	1		4	3	2	1
	2		5	3	2	
	3		4	5	1	
5.30	1			5		5
	2			6	1	3
	3			6	3	1
6.00	1				2	8
	2				4	6
	3			2	2	6
6.30	1					10
	2					10
	3				2	8
7.00	3					10

more than that of the anterior. In table 8, one hundred and twenty hours after section, this has occurred and the susceptibility of all three lots is almost the same. Still later the susceptibility of the posterior pieces provided they undergo complete reconstitution, may become even greater than that of the anterior pieces.

4. The one-eighth pieces

The regions represented by the pieces are shown in figure 3. In tables 9 to 12 only the records of pieces 1 to 4 are given.

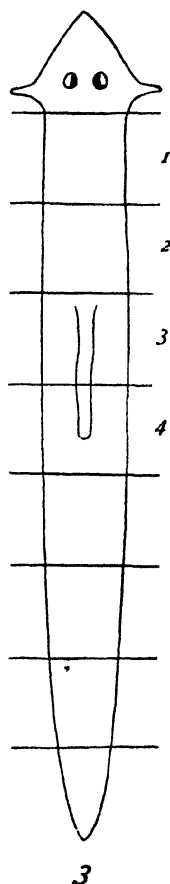
Table 9 compared with tables 5, 2 and 1 shows that in the first four one-eighth pieces the susceptibility is higher immediately after section than in the one-sixth pieces or one-fourth pieces or in the whole worms. Not only do these pieces complete disintegration earlier than the others, but the first traces of disintegration appear earlier.

After twelve hours, however, as shown in table 10, the susceptibility has decreased so that it is about the same as that of the whole animals. At twenty-four hours and also at forty-eight hours (table 11) it is still about the same. Seventy-two hours after section the susceptibility is again higher, at ninety-six hours still higher and at one hundred and twenty hours (table 12) it is higher still. Many of these short pieces in the more posterior regions do not undergo complete reconstitution but remain headless and in these the susceptibility does not undergo as great an increase as in those which produce wholes.

But the one-eighth like the one-sixth pieces show regional differences in the increase in susceptibility immediately following section. In table 9 it is evident that the anterior pieces (Lot 1) are in general least susceptible, the second eighths (Lot 2) considerably more susceptible, the third eighths (Lot 3) slightly more susceptible than the second, and the fourth eighth (Lot 4) about like the third. Here, as in the one-sixth pieces, the regions of the body which in the uninjured animal have lower susceptibility show a higher susceptibility after section, that is, the stimulation resulting from section is least in the anterior piece and increases

from piece to piece in the posterior direction except in the last two pieces where there is little difference.

After twelve hours (table 10) this stimulation has disappeared and now the susceptibility decreases from piece to piece in the posterior direction, i e., the original axial gradient has reappeared.



At twenty-four hours the relative susceptibilities of the one-eighth pieces remain about the same as at twelve hours. In table 11 (forty-eight hours) some slight irregularities in the susceptibility of different pieces appear. Lot 1 is most susceptible, Lot 2 less

TABLE 9

Susceptibility to KCN 0.001 m. of one-eighth pieces immediately after section

LENGTH OF TIME. IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
2 00	1	10				
	2	6	4			
	3	7	3			
	4	10				
2 30	1	10				
	2	3	7			
	3	4	5	1		
	4	6	4			
3 00	1	7	3			
	2	2	5	3		
	3	4	5	1		
	4	4	5	1		
3 30	1	6	4			
	2		8	2		
	3		5	4	1	
	4		6	4		
4 00	1	3	7			
	2		6	3	1	
	3		4	2	2	2
	4		4	2	2	2
4 30	1		6	4		
	2		1	4	4	1
	3		2	3	2	3
	4			3	5	2
5.00	1			9	1	
	2			3	2	5
	3			1	4	5
	4			2	1	7
5.30	1			6	4	
	2				3	7
	3				2	8
	4				2	8
6.00	1			1	9	
	2	-----	-----	-----	-----	10
	3	-----	-----	-----	-----	10
6.30	4	-----	-----	-----	-----	10
	1				5	5
7.00	1	-----	-----	-----	-----	10

TABLE 10

Susceptibility to KCN 0.001 m. of one-eighth pieces twelve hours after section

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
4 00	1	9	1			
	2	10				
	3	8	2			
	4	10				
4 30	1	8	1	1		
	2	9	1			
	3	8	2			
	4	9	1			
5 00	1	4	3	2	1	
	2	5	3	2		
	3	5	2	3		
	4	8	1	1		
5 30	1	1	4	3	2	
	2	4	2	3	1	
	3	5	1	2	2	
	4	6	2	2		
6 00	1	1	1	5	2	1
	2	3	1	2	3	1
	3	3	1	3	2	1
	4	4	2	2	2	
6 30	1	1	1		4	5
	2	2	2		4	2
	3		2	2	4	2
	4	3	1	1	2	3
7 00	1			1		9
	2	1		3	1	5
	3		1	1	3	5
	4	1		1	5	3
7 30	1					10
	2		1	1	1	7
	3				2	8
	4	1		2		7
8 00	2		1			9
	3					10
8 30	4	1				9
	2			1		9
9.00	4			1		9
	2				1	9
9.30	4					10
	2					10

TABLE 11

Susceptibility to KCN 0.001 m. of one-eighth pieces forty-eight hours after section

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
3 30	1	9	1			
	2	10				
	3	10				
	4	10				
4 00	1	7	3			
	2	10				
	3	10				
	4	10				
4 30	1	6	4			
	2	10				
	3	9	1			
	4	10				
5 00	1	4	5	1		
	2	10				
	3	9	1			
	4	10				
5 30	1		7	3		
	2	7	3			
	3	5	4	1		
	4	9	1			
6 00	1		4	3	3	
	2	4	6			
	3	4	2	3	1	
	4	8	2			
6 30	1		2	3	4	1
	2	4	3	3		
	3	1		5	3	1
	4	2	8			
7 00	1			3	2	5
	2	1	6		3	
	3		1	2	2	5
	4	1	6	1	2	
7 30	1			1	3	6
	2	1	3	3		3
	3			2	3	5
	4		3	4	2	1
8 00	1	-----				
	2		1	3		6
	3				1	9
	4		1	1	2	6
8 30	2			1	1	8
	3				1	9
	4				1	9
	2				1	9
9 00	3				1	9
	4	-----				
9 30	2	-----				
	3	-----				

susceptible, Lot 3 more susceptible than Lot 2, and Lot 4 again less susceptible than Lot 3. These irregularities are undoubtedly incidental and due either to differences in size of pieces, too large pieces showing too low and too small too high a susceptibility, or to stimulation of some of the pieces which cannot always be avoided and which distinctly increases susceptibility.

TABLE 12

Susceptibility to KCN 0.001 m of one-eighth pieces one hundred and twenty hours after section

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
3.30	1	7	3			
	2	8	2			
	3	9	1			
	4	8	2			
4.00	1	1	9			
	2	1	9			
	3	5	5			
	4	5	5			
4.30	1		5	5		
	2	2	4	4		
	3	4	4	1	1	
	4	4	4	1	1	
5.00	1			6	2	2
	2		2	2	2	4
	3	2	4	2		2
	4	3	4	1	1	1
5.30	1				6	4
	2			1	1	8
	3		3	3		4
	4		2	3		5
6.00	1					10
	2			1		9
	3			1	3	6
	4			3		7
6.30	2					10
	3				2	8
	4				3	7
	3					10
7.00	4					10
	4					10

At seventy-two and ninety-six hours the gradient in susceptibility from piece to piece is essentially that of the uninjured animal. At one hundred and twenty hours the same is true, as table 12 shows. Here Lot 1 has the greatest susceptibility, Lot 2 is next, Lot 3 next, and Lot 4 about like Lot 3. One-eighth pieces from the posterior region of the first zoöid undergo but little reconstitution and almost never produce whole animals while in the more anterior pieces the frequency of complete reconstitution is greater, consequently the differences in susceptibility in table 12 one hundred and twenty hours after section are due in part to a greater degree of reconstitution in anterior than in posterior pieces.

5. The one-twelfth pieces

The difficulty of cutting even large individuals into as many as twelve pieces of anything like equal size sets a limit to the study of consecutive series of pieces. It is of course possible to cut single pieces much smaller than this with a considerable degree of accuracy but in consecutive series the difficulties are much greater.

Figure 4 indicates the regions represented by the first six pieces which make up the first zoöid. Tables 13 and 14 give the susceptibilities immediately and forty-eight hours after section.

The susceptibility of all the pieces is very high immediately after section, being higher than that of any of the larger pieces (tables 2, 5, 9) or of the whole worms (table 1). The survival-time of even the least susceptible pieces is only half that of the whole worms and larger pieces. These short pieces are evidently more stimulated by the act of section than are larger pieces. After forty-eight hours, however, (table 14) the susceptibility of all pieces has decreased greatly and is only slightly higher than that of uninjured animals. The effect of section has at least largely disappeared.

Among the one-twelfth pieces only the more anterior produce whole animals and these do not in all cases. The more posterior pieces usually remain headless. Consequently the later increase

TABLE 14

Susceptibility to KCN 0.001 m of one-twelfth pieces forty-eight hours after section

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
3.00	1	9		1		
	2	9				1
	3	9				1
	4	10				
	5	10				
	6	10				
3.30	1	9			1	
	2	7	2			1
	3	9				1
	4	10				
	5	10				
	6	10				
4.00	1	9				1
	2	7	1	1		1
	3	9				1
	4	10				
	5	9		1		
	6	10				
4.30	1	6	3			1
	2	4	3			3
	3	4	5			1
	4	6	4			
	5	8	1			1
	6	10				
5.00	1	2	4	3		1
	2	2		1	1	6
	3	3	4		2	1
	4	5	2	1	1	1
	5	8				2
	6	10				
5.30	1		2	2	2	4
	2		1	1		8
	3		2	4	3	1
	4	5				5
	5	7		1		2
	6	7	3			
6.00	1			4		6
	2		1			9
	3			1	2	7
	4	2	3			5
	5	6	1			3
	6	6	1	1	2	

TABLE 14—Continued

LENGTH OF TIME IN KCN	LOTS	STAGES OF DISINTEGRATION				
		I	II	III	IV	V
6.30	1				1	9
	2			1		9
	3				1	9
	4		3	2		5
	5	5	1			4
	6	3	3		2	2
7.00	1					10
	2					10
	3					10
	4			2	1	7
	5	2	1	2		5
	6		2	4		4
7.30	4				2	8
	5			1	2	7
	6				2	8
8.00	4					10
	5					10
	6					10

III. CONCLUSIONS

A number of conclusions which will be shown later to be of the greatest importance for an understanding of the process of reconstitution are to be drawn from the data presented in the tables above. These are briefly stated and discussed in the following paragraphs.

1. *A temporary increase in rate of metabolism, a 'stimulation' lasting at least several hours, results from the act of section.*

Such a stimulation is to be expected for the act of section severs nerve cords and of course produces extensive injury to other tissue. Susceptibility determinations of pieces three hours after section, of which the records are not given, show that at that time the susceptibility is as high as immediately after section. From this time on it gradually decreases and twelve hours after section the susceptibility of the pieces is about the same as that of the uninjured animals.

This also is to be expected, for the piece is no longer in connection with its chief sources of stimulation, that is, first the head region and secondly other parts of the body. As regards movement it is much less active than the intact animal and if it were not for the wounds at its ends and the growth processes beginning there its rate of metabolism would undoubtedly fall far below that of the uninjured animal.

2. The temporary increase in rate of metabolism following section varies in amount inversely as the length of the piece.

A comparison of tables 2, 5, 9 and 13 will show that in general the smaller the pieces the higher the susceptibility. The one-fourth pieces show in the tables practically no increase in susceptibility following section. As a matter of fact the regions adjoining the cut ends do show an increased susceptibility but it does not involve the piece as a whole.

That the shorter piece should be more stimulated than the longer by section is also in accord with expectation. The shorter the piece the more direct the injury and resulting stimulation of its conducting paths and tissues. In very short pieces almost the whole length becomes involved in the wound reaction at the two ends. Tables 10 and 14 show that the susceptibility of the one-eighth and one-twelfth pieces does not fall during the forty-eight hours following section to so low a level as that of the longer pieces. This difference is undoubtedly due to the fact that in the shorter pieces the wound reaction affects the whole rather than merely the two terminal regions as in the longer pieces. In the long pieces the susceptibility of the two terminal regions is distinctly higher than that of the rest of the piece at this stage.

3. The temporary increase in rate of metabolism following section varies in amount according to the region of the original body which the piece represents, being least in the most anterior and increasing in successively more posterior pieces.

This regional difference in stimulation is associated with length of piece and is not apparent in the long one-fourth pieces, but in the one-sixth, one-eighth and one-twelfth pieces it is distinct. The rate of metabolism in the first zoöid of uninjured animals is highest in the anterior region and decreases more or less regularly

in the posterior direction (Child '13 c). In the one-fourth pieces of table 2 this gradient continues to exist after section, the rate of the second fourth being lower than that of the first. In some other series the rates of first and second fourths immediately after section are about the same, that is, the original gradient has disappeared. In the one-sixth, one-eighth and one-twelfth pieces (tables 5, 9 and 13) we see that in general the susceptibility increases from piece to piece posteriorly, i. e., the original axial gradient is reversed. This can be due only to the fact that more posterior pieces are more strongly stimulated by section and this is actually the case. A comparison of the susceptibilities of Lot 1, which represents the most anterior fourth, sixth, eighth or twelfth in tables 2, 5, 9 and 13, shows that the susceptibility of this anterior region is but little affected by length of piece and undergoes but little increase after section until we reach the one-twelfth pieces, where the increase is greater than in the longer pieces. The more posterior pieces are much more affected by section and the tables show that in general the more posterior the level of a piece the greater the degree of stimulation following section.

As regards the regional differences in rate immediately after section, the results of the susceptibility method have been confirmed by estimations of CO_2 production very kindly made at my request by Dr. Tashiro with the apparatus devised by him (Tashiro '13). In one-fourth pieces or larger the second piece shows a lower rate of CO_2 production than the anterior. In one-sixth pieces the anterior piece has the lowest rate of CO_2 production and the rate increases posteriorly from piece to piece.

This regional difference in degree of stimulation agrees with the wellknown fact of observation that when such animals as earthworms and planarians are cut in two the posterior piece usually reacts much more strongly than the anterior and it is also in full accord with the theory of antero-posterior dominance developed in preceding papers (Child '11 d, '13 b, '13 c), in fact it constitutes valuable experimental evidence for this theory.

The greater stimulation of posterior as compared with anterior regions in consequence of section can only mean that the rate of

metabolism in the anterior region is to a greater degree independent of conditions in other parts of the body and so is but little altered when conducting paths are cut. The rate of metabolism in the posterior region on the other hand, must be in high degree dependent upon its connection with other regions, for when this connection is severed the stimulation of conducting paths increases its rate very greatly. The more anterior levels of the body are largely independent of the posterior while the more posterior levels are largely dependent upon the anterior. The degree of independence or subordination of a body-region then depends upon its level in the body.

There is thus an axial gradient in degree of stimulation resulting from section with its lowest point at the anterior and its highest point at the posterior end and this is evidently the expression of a gradient in degree of subordination.

That these gradients are expressions of the axial gradient in rate of metabolism described in the preceding paper (Child '13 a) cannot be doubted. It was pointed out in that paper that the region of highest metabolic rate in a living cell or cell aggregate must be much more independent of other regions than they are of it and so must inevitably dominate other parts to a greater or less extent and within a greater or less distance from it. In *Planaria* where a simple axial gradient in rate from the head posteriorly exists the head is unquestionably the dominant region. As regards functional relations this is self-evident but it is also true at least for certain features of morphogenesis (Child '11 d). As pointed out above, the stimulation gradient in pieces is merely an expression under special conditions of the axial gradient and of antero-posterior dominance. The increase in stimulation from section with increasing distance from the anterior end means simply increasing dependence upon stimuli coming from more anterior regions.

4. *A remarkable relation between frequency of head formation and degree of stimulation after section exists. The greater the increase in rate of metabolism after section the less the frequency of head-formation.*

In the first paper of this series (Child '11 c) it was shown that the frequency of head-formation *decreases* as the length of the piece decreases and also from anterior to posterior regions. In the present paper we have seen that the degree of stimulation *increases* as the length of the piece decreases and also that the degree of stimulation is greater in posterior than in anterior pieces. It would seem that these two features, degree of stimulation after section and frequency of head formation, must be in some way associated or have a common foundation. But before the relation between them can be made clear it is necessary to find when in the history of a piece it is determined whether the piece shall give rise to a head or not. And consideration of this problem, the time of head determination, must be postponed to another paper. The chief purpose of the present paper, however, is to point out this inverse relation between head frequency and degree of stimulation after section. The following paper will afford an insight into the nature of the relation.

5. *After the temporary increase in rate of metabolism following section has disappeared a second slow and (relatively) permanent increase in rate occurs, in connection with reconstitution and this is a process of rejuvenescence resulting from the reorganization and reduction of the pieces.*

The writer has called attention elsewhere (Child '11 a, '13 d) to the occurrence of rejuvenescence in connection with experimental and asexual reproduction in planarians. The susceptibility tables in the present paper show merely the earliest stages of this process at one hundred and twenty hours after section (tables 4, 8, 12). This slow increase in rate in the pieces, which begins after three or four days and which indicates the beginning of rejuvenescence is relatively permanent, i. e., it disappears only gradually as the animal undergoes growth and becomes older in consequence of feeding. The degree of rejuvenescence is in general inversely proportional to the size of the piece and directly proportional to the degree of reconstitucional change. In this connection another expression of the axial gradient in rate of metabolism may be mentioned, although it is not clearly shown in the tables. In general, the degree of rejuvenescence in recon-

stitution is greater in posterior than in anterior pieces of the same size, provided of course that both produce new whole animals. The amount of reorganization in the reconstitution of a piece undoubtedly increases as the level of the piece becomes more posterior. The amount of new tissue behind the head and the degree of change in the alimentary tract both increase from anterior to posterior pieces. In other words, the farther from the head a given region of the body is, the greater the alteration it must undergo when brought into direct relation with a dominant head region as every piece is when a head forms at its anterior end.

IV. SUMMARY

1. A temporary increase in rate of metabolism, a 'stimulation' lasting at least several hours, results from section.

2. The temporary increase in rate of metabolism following section varies in amount inversely as the length of the piece, long pieces being very slightly or not at all stimulated and short pieces strongly stimulated.

3. The temporary increase in rate of metabolism following section also varies in amount according to the region of the body which the piece represents, being least in most anterior and increasing in successively more posterior pieces. In the shorter pieces this difference is sufficient to increase the rate of posterior above that of anterior pieces.

4. The differences along the axis in the stimulation from section are merely special expressions of the axial gradient and the antero-posterior dominance which results from it.

5. An inverse relation exists between the frequency of head formation and degree of stimulation of pieces following section. The shorter or the more posterior a piece the greater the degree of stimulation and the less the frequency of head formation. The nature of this relation will be considered after presentation of further facts.

6. The temporary increase in rate of metabolism in the piece after section is followed by a gradual fall. Twelve hours after section the rate of metabolism in the pieces may be as low as, or

lower than, that in corresponding regions of intact animals. During the following thirty-six to forty-eight hours the rate of metabolism remains about the same in the pieces.

7. After three or four days the rate of metabolism in the pieces begins to rise slowly as reconstitution proceeds. This rise in rate is relatively permanent and constitutes physiological rejuvenescence. It disappears only as the animal increases in size and becomes older with feeding. Its amount depends upon the degree of reconstitucional change and the size and region of the piece. Provided complete reconstitution occurs the degree of rejuvenescence is greater in posterior than in anterior and in small than in large pieces.

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ON THE STRENGTH AND THE VOLUME OF THE WATER CURRENTS PRODUCED BY SPONGES¹

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Ever since Grant discovered the currents produced in water by living sponges, this activity has been a matter of interest to the naturalist. The arrangement of the canals within the body of the sponge, the disposition of the motor elements or choanocytes, and the control of currents by means of sphincters have all received much consideration. Very little attention, however, has been given to the currents themselves, their strength and volume.

In a paper published in 1910 in the eighth volume of *The Journal of Experimental Zoölogy*, I recorded the fact that in *Stylotella*, a sponge from the coast of North Carolina, the pressure of its currents was equal to that of a column of water from 3.5 to 4 mm. in height. This sponge is of small size and generates only a slight disturbance in the surrounding water as compared with what may be seen in the vicinity of large sponges in the tropical and subtropical seas. Here the water often wells up so abundantly from the sponge as to deform the surface of the sea much as a vigorous spring deforms that of a pool into which it issues. Sponges of this description are frequently met with in the waters about the Bermuda Islands, and, during a recent sojourn at the Bermuda Biological Station, I took the opportunity of measuring the currents from some of these organisms.

The method that I used in making these measurements was the same as that which I had previously employed for *Stylotella*. A whole sponge or a large portion of one was transported in a tub or bucket to the laboratory and a glass tube of appropriate size was tied securely into one of its oscula. Care was taken to select for this purpose an osculum whose canal system had been unin-

¹ Contributions from the Bermuda Biological Station for Research, No. 32.

jured in detaching the sponge from its natural base or, if it had been necessary to break open the canal system anywhere, such breaks were closed by ligatures of soft string. The tube with the sponge attached was then held rigidly in place in the bucket of water and, after five minutes or so, the height of the water in the tube over that in the bucket was measured by means of a millimeter rule attached to the side of the tube. Without disturbing the tube and its attached measure, the canal system of the sponge was then cut open so that the water in this system was in free communication with that in the bucket. The water in the tube immediately fell and, after it had come to a constant level,

TABLE 1

Showing pressures in millimeters of seawater exhibited by the currents of seven species of sponges from the Bermuda Islands

NAME OF SPONGE	HEIGHT OF COLUMN OF SEAWATER IN MILLIMETERS FOR FIVE DETERMINATIONS					AVERAGE HEIGHT
<i>Tethya</i> sp. Close to <i>T. seychellensis</i> (E. P. Wright).....	2.5	2.0	2.5	2.0	2.0	2.2
<i>Spirastrella</i> sp. Close to <i>S. vagabunda</i> , var. <i>tubulodigitata</i> Dendy.....	2.5	2.0	2.0	2.5	2.0	2.2
<i>Pachychalina</i> sp.....	1.0	2.0	1.5	1.0	1.0	1.3
<i>Spinoseella</i> <i>sororia</i> (Duch. et Mich)	3.0	2.5	3.0	3.0	3.0	2.9
<i>Tedania</i> sp.....	2.0	2.0	2.5	2.0	2.0	2.1
<i>Stelospongia</i> sp.....	2.5	2.0	2.0	2.0	2.0	2.1
<i>Hircinia variabilis</i> F. E. Schulze..	3.0	3.0	2.0	2.5	3.0	2.7

a reading of its height was again taken. The difference between this reading and that made before the sponge was cut open was assumed to be a measure of the pressure of the water current produced by the sponge.

Determinations of the current pressures by the method just described were made in seven species of sponges, and the details of of these measurments are given in table 1. At least five determinations were made for each species. I am under obligations to my friend, Prof. H. V. Wilson for the identification of the sponges upon which these determinations were made.

The surprising feature of the determinations in the table is that they all indicate a very low current pressure, lower even than that

of *Stylotella*. Notwithstanding this general lowness of pressure one of these species, *Spinosella sororia*, the common Bermuda finger-sponge, produces in the shallow sea a conspicuous deformation of the natural surface of the water, and this deformation can also be observed in specimens that have been transported to the laboratory. As *Spinosella* was a very satisfactory species to work with, a determination of the volume of water that passed through a single finger of this sponge was attempted. This was made by sinking the tube that had been fastened into the osculum of a finger of *Spinosella* under the surface of the seawater and thus allowing the water driven by the sponge to escape. The escaping current deformed the surface of the water in the bucket in much the same way as is often seen over a vigorous sponge finger in its natural position in the sea. The rate of the flow of water from the finger was then determined by measuring the velocity of floating particles, such as carmine and so forth, that were carried up the tube by the water current. This proved to be very close to 20 mm. in five seconds. As the diameter of the tube was 17 mm. the finger must have discharged a little over 4.5 cc. of water in five seconds. At this rate the discharge would amount to some 78 liters a day. This finger measured about 10 cm. in length and averaged 4 cm. in diameter; its osculum had a diameter of about 2 cm. *Spinosella* colonies often consist of as many as twenty such fingers and, assuming that all the fingers work at the same rate as the one upon which the measurements were made, such a colony as a whole would strain in a day about 1575 liters of seawater or over 415 gallons.

Since all the measurements recorded in the table were made upon sponges that had been removed from their natural attachments and transported to the laboratory and since the effect of such treatment is known to retard rather than accelerate the currents, the results therein recorded are believed to be below rather than above the actual working capacity of the several sponges. This opinion is borne out by an observation made on *Spirastrella* for which I am indebted to Mr. W. J. Crozier and Mr. D. H. Wenrich. A specimen of this sponge was found partly exposed at low water. Although the osculum was out of water, there was

still a free flow from it. A cut was made on the side of the sponge at the level of the outer seawater and the sponge was taken to the laboratory. Close measurements showed that the edge of the osculum over which the water lifted by the sponge had been flowing was 4 mm. above that of the sea level, demonstrating that this sponge in the undisturbed condition in which it was found had been overcoming a pressure of 4 mm. of seawater. As the highest pressure that had been obtained from *Spirastrella* in the laboratory was 2.5 mm., it seems probable that the laboratory determinations for all the species tested are a little below the actual maxima for this form of activity, though, for reasons already given in my former paper on *Stylotella*, they cannot be much below this maximum.

From these observations it is clear that the currents produced by sponges consist of relatively large volumes of water flowing at low pressure and that currents of this kind are capable of producing such deformations of the surface of the sea as can be observed above large sponges. It would be interesting to ascertain what proportion of the suspended material in the seawater is screened out as the current passes through the sponge, but no observations on this point were made.

MODES OF INHERITANCE IN TELEOST HYBRIDS

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THIRTY-EIGHT FIGURES

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INTRODUCTION

The present contribution has a twofold purpose, to review and discuss the literature on teleost hybrids and to present a new body of experimental data, which seem to throw added light on various mooted questions and to lead to rather definite conclusions concerning certain problems of inheritance.

As material especially available for the study of the early stages of hybrid development the teleosts are unexcelled by any

other group of animals known to the writer, although a much larger number of investigators have chosen to work with echinoderms. Teleosts possess decided advantages over the echinoderms in that they intercross in practically any combination and without the aid of the reagents used in artificial parthenogenesis, which are necessary in order to cross echinoderms, and which may affect the character of development and thus interfere with the study of heredity. It is, moreover, a simple matter to rear the young of teleosts to a stage in which many definitive characters can be studied, while hybrid echinoderms have never to my knowledge been reared beyond a comparatively early larval stage. In addition to these two fundamental advantages teleosts afford especially good material for this type of investigation because the large number of species in any neighborhood give a very wide range of possible combinations; because the eggs and young are large and easily handled; and because the contrasting characters are more numerous and more clearly defined than are those of echinoderms.

HISTORICAL REVIEW OF THE WORK OF APPELLÖF, MOENKHAUS, KAMMERER, BANCROFT AND NEWMAN ON TELEOST HYBRIDS

The earliest published work on teleost hybrids that has come to my attention is an important paper by Appellöf that appeared early in 1894. I regret that this paper escaped my notice until after the publication of my two former papers on *Fundulus* hybrids, for it would have been highly suggestive for certain phases of my own work. The paper, although rather generally overlooked by investigators, should be of considerable interest to all those who are engaged in the study of the developmental mechanics of heterogenic hybrids. Appellöf was probably the first to attempt crosses between very distantly related species. His combinations include such ill-assorted matings as the stickleback and the flounder, the cod and the cunner, the cod and the flounder and others equally bizarre. In each case he found that, even though a mature hybrid between the two parent types be unthinkable, development in the cross fertilized eggs proceeds

for a considerable period, in most cases through the cleavage period, in a normal manner. In all cross bred eggs the cleavage seemed to be identical with that of pure bred eggs except that in rare cases the time rate of the cleavage process was markedly retarded. This was especially noticeable in the hybrids from *Spinachia* eggs fertilized with *Gasterosteus* sperm.

An observation of Appellöf's, which to my mind is of considerable morphological significance, is to the effect that the most critical period in the development of heterogenic hybrids is the period of transition from cleavage to germ layer formation—the period of 'gastrulation.' In comment upon this finding I may say that my own results have for a number of years forced me to similar conclusions and that I have on hand an investigation dealing at some length with this point. It is of more than passing interest to note how this idea lines up with the rather prevalent opinion that the process of cleavage is purely a function of the egg, and that the spermatozoön, though coöperating with the egg nucleus in the mitoses of cleavage, does not begin to influence the process of heredity until during the formation of the gastrula. It would appear that even a very distantly related sperm may stimulate development in an egg and coöperate harmoniously with the latter through the period of cell multiplication, but that when the hereditary functions of the germinal elements come into play discordant chemical interactions take place that effectually disrupt developmental harmony. This barrier to further development I have found to be less sharply drawn than Appellöf supposed, as will become evident when the data subsequently presented in this paper claim the reader's attention.

Next in chronological order comes the work of Moenkhaus ('94) in which he relates the results of his crosses between *Fundulus heteroclitus* and *Menidia notata*. This work had as its object the study of the behavior of maternal and paternal chromatin in hybrid eggs and embryos in which the chromosomes of the parental species were visibly different in form. The observations show that the paternal chromosomes retain their identity in the zygote and for a considerable period remain in a close

group sharply marked off from the maternal group. Subsequently, however, the two types intermingle, though still retaining their specific characters. The rate of cleavage was, according to Moenkhaus, the same as in pure bred eggs of the same species. It is a noteworthy fact that when *Fundulus* eggs are fertilized with *Menidia* sperm, the period of gastrulation is safely passed and a well-defined, though short and defective embryo is formed although the two species represent two distinct orders of teleosts.

Kammerer ('07) published a rather extensive account of hybridizing two species of perch, *Perca fluviatilis* and *Acerina cernua*. In brief his report is as follows. In the spring of 1905 a fisherman brought to the laboratory two specimens of an unknown type of perch, which, at the suggestion of Doctor Przibram, were adjudged to be hybrids between *Perca fluviatilis* and *Acerina cernua*, because in a number of respects they appeared to be intermediate between these two species. In order to test the correctness of this diagnosis it was decided to intercross the hypothetical parent species in order to determine whether the hybrids resembled the questionable wild individuals. It was found that the two species did cross successfully and that a number of young fish hatched and grew well for about six months, at which time they showed some characters which slightly resembled those of the supposed adult wild hybrids. The latter were successfully crossed, since they were both fertile females, with males of both *Perca* and *Acerina*, the young resembling closely the paternal species in each case. This is the only case on record, so far as I am aware, of fertile hybrids being produced by crossing two genera. It is rare, in fact, to find undiminished fertility in hybrids between two closely related species of the same genus, and even inter-varietal hybrids frequently show incomplete fertility. Had Kammerer reared to maturity and successfully bred the unquestioned hybrids which he obtained he would have made good his case, but as the matter stands there is considerable cause to doubt the validity of his conclusions.

The chief value of the paper in the present discussion lies in the fact that there is presented a considerable array of data,

tending to show that in practically all characters examined the hybrids reared in the laboratory are strictly *intermediate* between those of the parent species. This is true for such matters as numbers of scales in a row, numbers of fin-rays, numbers of teeth, and other integral variates. Characters involving dimensions show a similar *blending* between the two species. No mention is made of the discovery of any type of inheritance suggestive of Mendelian dominance.

In addition to the crosses mentioned above Kammerer made a number of heterogenic crosses between teleost species, the results of which led him to conclude that the degree of success in the development of hybrids depends not upon the closeness of relationship of the parent species but upon their similarity of habitat. The fallacy of this conclusion is evident in view of the success of Moenkhaus and the writer in cross fertilizing practically any two species of teleosts.

The first attempt to follow in detail the development of teleost hybrids was made by the writer (Newman '08). This paper dealt exclusively with the reciprocal hybrids between *Fundulus heteroclitus* and *Fundulus majalis*. The former species develops nearly twice as rapidly as the latter and the reciprocal hybrids both have a developmental time rate intermediate between those of the parent species, though more nearly like that of the egg than that of the sperm species. All characters involving the time factor show blended inheritance, but many characters not dependent on the time factor were shown to exhibit more or less typical Mendelian dominance. Some of the dominant characters noted were as follows: *F. heteroclitus* type of chromatophore dominant over that of *F. majalis*; size of young on hatching or at the time of maximum development that of *F. heteroclitus* in both reciprocal crosses; the rate of cleavage nearly pure maternal; the viability of young hybrid larvae, when capable of hatching, equal to or greater than that of the more viable species, *F. heteroclitus*; resistance to lack of oxygen or presence of carbon dioxide of *F. heteroclitus* egg hybrid as great or greater than that of the more resistant species; susceptibility to these reagents greater in *F. majalis* egg hybrids than in the more susceptible

species, *F. majalis*; the depth of pigmentation of young *F. heteroclitus* egg hybrids as pronounced or more so than the darker species, *F. heteroclitus*. These cases in which the hybrids seemed to show an exaggeration of dominance exemplify the phenomenon of 'hyperdominance.' In view of the fact that at the time when this paper was written the new Mendelism was sweeping all before it, I took pains to emphasize especially all types of inheritance which appeared to me to be essentially non-Mendelian. This stand was taken advisedly in the hope of checking the growing impression that all inheritance was on analysis Mendelian, and that cases of apparent blending, even in the F_1 generation of hybrids, was the result of incomplete analysis of the factors involved.

Chief stress was laid upon the importance of studying heredity as a *process*, of observing the genesis of characters rather than limiting observation to the definitive conditions as they appear in adults. In this connection it was noted that maternal and paternal influences showed alternating periods of predominance, so that a character might appear as a dominant at one time only to be superseded by the character that had apparently been recessive. The definitive condition represents only the end result of a struggle between opposed parental tendencies whose ups and downs may be observed from day to day in developing hybrid embryos.

The question as to whether the foreign sperm exercises any influence upon the rate and character of early cleavage was in this paper answered in the negative for the reason that in all early experiments the differences were very small and not always in the same direction.

This paper was followed nearly two years later by another dealing with the same species of fish (Newman '10), in which the question as to the influence of the spermatozoön on early development was reëxamined. Very searching statistical methods were applied to the relative rates of early cleavage in pure and hybrid *F. majalis* eggs. It was found as the result of five experiments involving very large numbers of eggs, that there was a slight but constant acceleration of cleavage as the result of the

use of foreign sperm. On this score I took occasion to criticise the position of Conklin, Godlewski and others, who, on the basis of the work done on echinoderm hybrids, had declared that the early development of hybrids is purely maternal and that it is only in stages later than the gastrula that the influence of the spermatozoön begins to make itself felt. There was in my experiments a real effect of foreign sperm on the rate of early development and this effect was not due to a more speedy impregnation of the egg membrane by the foreign sperm head, as was shown by the experiment in which all spermatozoa in both hybrid and control cultures were destroyed with distilled water within less than five minutes after insemination. By this time all spermatozoa had entered the eggs, those of the foreign and native species having entered with equal promptness.

These results were criticised by Godlewski ('11) because they were not confirmed by cytological studies. He did not explain, however, just what he meant by a cytological confirmation of a statistical treatment involving many thousands of eggs, no small sample of which could be expected to show anything of value. On the basis of what seemed to be a demonstration that foreign sperm actually effects a slight change in the rate of early cleavage, the writer entered upon a general discussion of the rôle of the spermatozoön in early development and came to the conclusion that, in addition to initiating development, the paternal germ plasm reacts almost immediately with that of the egg so as to change the rate and character of metabolism and thus accelerate or retard cleavage. Naturally the cytoplasmic organization of the egg imposes upon the young embryo certain morphological restrictions, but in all those matters that have to do with rate of chemical reaction the spermatozoön begins to exert an influence as soon as fertilization occurs, as soon as the two ontogenies are engrafted one upon the other. The work of Godlewski, Baltzer and others, who had^e experimented with echinoderm hybrids, was cited by the writer in confirmation of his conclusions and it is to be regretted that, in citing Godlewski's results certain misstatements were made, for it was in response to these inadvertent inaccuracies that Godlewski took the whole

paper rather severely to task. The fundamental contention was, however, scarcely touched upon by this critic, as was shown by the writer (Newman '11) in a reply to Godlewski, in which the minor inaccuracies in reporting the latter's results were admitted and differences of interpretation were brought clearly upon a controversial plane.

It may then be considered as fairly well established that, *at least in the case of hybrids between closely related species, the spermatozoön exercises at first a slight and then a progressively more pronounced influence upon the rate of early development, but fails to exercise any really hereditary function until the embryo begins to differentiate tissues and organs.* This point of view will, I believe, be justified by the new data herewith presented. This somewhat radical change of front on my part is the result of several years of study and has been forced upon me especially by the experiments about to be reported, the results of which are herewith to some extent anticipated.

In the Proceedings of the Indiana Academy of Science for 1910, published a year later, Moenkhaus reports upon an extensive series of experiments dealing with "Cross fertilization among fishes." These range from extreme heterogenic crosses between different orders of teleosts to those between closely related species of the same genus. A useful summary of the chief facts and conclusions is furnished and is here quoted:

1. The eggs of any of the species of teleosts tried may be impregnated by the sperm of any other species tried.
2. The number of eggs fertilized is usually great, i.e., 75 per cent or more. This bears no relation to the nearness of relationship of the two species concerned.
3. Normal impregnation is the rule, di- and polyspermy being the exception.
4. Development in its early stages proceeds normally, the deleterious effects of the two strange sex products upon each other showing only at later cleavage or subsequently.
5. The rate of development in the early cleavage stages is always that of the egg species. Any effect of the strange sperm upon the rate of development shows itself by slowing the process regardless of whether the rate of the sperm species is faster or slower than the egg species. A period of great mortality in the developing hybrids is gastrulation.

6. If the heart is formed, although it pumps no blood, the embryo may remain alive for a considerable period, yolk absorption taking place to a varying degree. If the heart handles blood and the blood vessels are differentiated, the embryo is likely to develop to the point of hatching.

7. The numerous abnormalities appearing in the hybrid embryos are due to a deterioration in the developmental processes, resulting probably from the poisonous action of the sex products upon each other.

8. The success of the hybrids, i.e., the stage to which a given hybrid will develop, is correlated with the nearness of relationship of the two species used.

9. The mixing of unrelated sex products is looked upon as analogous to the transfusion of unrelated bloods, the more distantly related the two species concerned the greater their toxicity.

For the most part the results of Moenkhaus confirm the earlier results of Appellöf and extend the horizon of our knowledge of the field in question. I am unable, however, to accept any of the last six conclusions listed above, and will present my reason for thus dissenting after presenting my new results.

Bancroft ('12) has made the most recent contribution to the literature on the hybridization of teleosts. He repeated the experiments published by the present writer in 1908, intercrossing *Fundulus heteroclitus* and *F. majalis*, paying especial attention to the heredity of pigmentation. The avowed object of the experiments was to reëxamine the hybrids "from the Mendelian and physiological points of view." Bancroft confirms my observations and in most points agrees with my conclusions. In his search for evidences of Mendelian dominance he overlooks many *intermediate* conditions. In one instance he attempts to explain what had seemed to me a perfect case of blended inheritance as the result of the interaction of two separate unit factors. He confirms my results and conclusions very exactly when he concludes that "in general, characters connected with rate of development show blended heredity and it may be that such characters are so intimately associated with extra-nuclear substances such as the yolk that complete dominance is not obtained."

It is noteworthy, in view of Moenkhaus's statement "that any effect of the strange sperm upon the rate of development shows itself by slowing the process of development regardless of whether

the rate of the sperm species is faster or slower than the egg species," that Bancroft takes the position that:

As regards the rate of development of the embryos my observations confirm those of Newman on most points. The development of the *F. heteroclitus* egg hybrid was shown to be slower than that of its maternal parent; and the development of the *F. majalis* egg hybrid, during the early stages, was faster than that of the pure *F. majalis*. After hatching the *F. heteroclitus* egg hybrid seemed more vigorous and grew faster under like conditions than either of the pure forms.

It is somewhat strange that Moenkhaus failed to note these facts for he must have performed the experiment a number of times. Bancroft's paper adds materially to our knowledge of the heredity of pigment in teleost hybrids. His analyses of these processes is more nearly adequate than that of any other writer. In the general discussion of new data I shall have occasion to come back to this paper for more detailed treatment.

This then is the history of the development of our knowledge of heredity in teleost hybrids. From this array of facts and theories it is possible to extract certain well-defined problems and unsettled questions, which it is my intention to put to the test of further experiment. A list of some of the more important questions is herewith introduced in order to clarify the issue and to focus the attention of the reader upon the problems involved.

1. At what point in the development of an embryo does the sperm begin to exercise an hereditary as opposed to a merely chemical effect upon the character of development?

2. Is the rate of cleavage 'inherited,' in a strict sense, or is it merely a function of egg size and of yolk content?

3. Is the effect of foreign sperm to be looked upon as necessarily deleterious, and hence toxic, or is the effect bad only when there arises so pronounced a disharmony of the two engrafted ontogenies that no blending or compromise can be reached?

4. Is it true that all characters concerned with or resulting from the rate of development are inherited in the blended fashion? Is rate of development strictly an hereditary character?

5. Is the degree of success in the development of hybrids in any way closely correlated with the nearness of relationship of

the two species used? If not, upon what factors does success in crossbreeding depend?

6. Are structural or physiological characters inherited in the exclusive or in the blended fashion in the first generation of hybrids?

7. What is the function of the spermatozoön in early development? Is its initial effect merely equivalent to that exercised by mechanical and chemical agents used in artificial parthenogenesis, or is this only part of its function?

8. Is there any truth in the analogy suggested by Moenkhaus between the effects resulting from mixing strange sex products and those resulting from the transfusion of unrelated bloods?

Each of these questions will receive its proper share of attention elsewhere in the paper.

NEW EXPERIMENTS: RECIPROCAL CROSSES BETWEEN SPECIES OF THE GENUS *FUNDULUS* AND THE GENUS *CYPRINODON*

1. MATERIALS AND METHODS

The four species of fish used in the present experiments belong to two genera of the family Poeciliidae and are common in the waters about Woods Hole. They are *Fundulus heteroclitus* (figs. 1 and 2), *F. majalis* (figs. 3 and 4), *F. diaphanus* (figs. 5 and 6), and *Cyprinodon variegatus* (figs. 7 and 8). *F. heteroclitus*, *F. majalis* and *Cyprinodon* are marine or brackish water species, while *F. diaphanus* is strictly a fresh water form though tolerant of slightly brackish water. In some regions *F. heteroclitus* is found with *F. diaphanus* in fresh water ponds into which the sea flows at high tide. The eggs of *F. majalis* are the largest, averaging about 2.7 mm. in diameter, those of *F. diaphanus* come next with an average diameter of 2.3 mm., those of *F. heteroclitus* average 2 mm., and those of *Cyprinodon* are much the smallest, averaging scarcely 1.5 mm. The yolk of *F. majalis* eggs is denser and of deeper yellow color than that of the other species; that of *F. heteroclitus* is denser and yellower than in *F. diaphanus* but less so than in *F. majalis*; that of *Cyprinodon* is almost colorless.

During the months of June and July all of these species are spawning in the Woods Hole region and little difficulty is experienced in obtaining abundant material for crossbreeding. The most successful experiments were those in which insemination was accomplished by the dry method. In this way it was possible to avoid exposing either of the sexual products to waters of harmful concentration, for eggs were stripped from well dried females into bowls containing no water and they were mixed with fresh milk or macerated testis. After allowing a few minutes for the sperm to impregnate the eggs, water of the proper kind was added and excess sperm washed out. It was found best to rear the eggs of *F. heteroclitus*, *F. majalis* and *Cyprinodon* in sea-water and those of *F. diaphanus* in fresh water. All thrive well in about 25 per cent sea-water, but do no better than when the two kinds of natural water are used. Likewise all four kinds of eggs thrive in fresh water, but the eggs of *F. diaphanus* do not do well in natural sea-water, in which they undergo various degrees of plasmolysis.

No less than three crosses of each type were performed and careful comparisons were made possible through the frequent camera drawings of individuals and details. The illustrations herewith published are taken from camera drawings which seem to represent the most typical condition. The method of presenting the data is as follows: A chronology is given for each cross and beside it in an adjacent column are given the details of synchronous stages in the development of the control or pure bred eggs of the maternal species. Only such details are mentioned as seem of particular significance as diagnostic of relative rates of development or types of inheritance involved. In order that repetition may be avoided the chronologies for the reciprocal hybrids between *F. heteroclitus* and *F. majalis* are omitted, and the reader is referred for the facts to the previous paper in which these crosses were first dealt with (Newman '08). The references to details of pigmentation are intended to be merely suggestive, since the study of pigment inheritance forms a special topic for subsequent discussion.

2. TABULATION OF DATA

a. Crosses between species of the same genus

TABLE 1

Comparing the hybrids from *F. diaphanus* ♀ × *F. heteroclitus* ♂ with pure bred *F. diaphanus*

TIME	PURE <i>F. DIAPHANUS</i> (CONTROL)	<i>F. DIAPHANUS</i> ♀ × <i>F. HETEROCLITUS</i> ♂
2 hours	First cleavage	Same
6 hours	Advanced cleavage	A larger proportion in more advanced stages than in control
20 hours	Blastoderm with germ ring faintly defined, embryonic shield barely visible (fig. 10)	(Germ ring well defined and well established embryonic shield (fig. 11)
2 days	Germ ring halfway around the yolk, embryonic axis well defined, no neural tube (fig. 13)	Germ ring nearly around yolk, embryo long and with brain and optic vesicles well defined (fig. 14)
3 days	Blastopore closed, embryo with short tail, lenses of eyes lenticular in form. Mid-brain broadly open, no heart-beat or circulation, first chromatophores under hind-brain and on adjacent yolk	Blastopore closed, tail long, lenses spherical and partially enclosed in optic cup, entire brain closed, heart beating in majority of embryos, much more numerous and better defined chromatophores on brain and yolk
4 days	Heart-beat beginning to be established in nearly all embryos, no circulation, scattering stellate dark chromatophores on top and sides of brain, black yolk chromatophores scattered over whole yolk, a few small red yolk chromatophores of dull orange color (fig. 16)	Heart beating strongly, yolk and body circulation well established, pigmentation much more advanced in every particular than in control, red yolk chromatophores redder than in control (fig. 17)
5 days	Embryos about like those of pure <i>F. heteroclitus</i> at 4 days, heart-beat strong, circulation well established, head chromatophores all brownish and small, large black yolk chromatophores arranged along the principal vitelline vessels, red yolk chromatophores large, stellate, and dull reddish brown	Embryos less markedly in advance of control than at 4 days, a few large black head chromatophores of the <i>F. heteroclitus</i> type have appeared along with numerous small brownish head chromatophores of the <i>F. diaphanus</i> type, red yolk chromatophores intermediate in color between the red-brown of the maternal and the brick red of the paternal species

TABLE 1—Continued

TIME	PURE <i>F. DIAPHANUS</i> (CONTROL)	<i>F. DIAPHANUS</i> ♀ × <i>F. HETEROCLITUS</i> ♂
1 week	Embryo as in fig. 19, almost even with the hybrid strain in stage of development, other details about as before	Embryos as in fig. 20, tail shorter than in control, other details as before
9 days	Embryos comparatively light colored, tail long and slender	Embryos much darker than control, decidedly larger and more advanced
12 days	None hatched	Nearly all hatched
13 days	A few hatched	All hatched and very active
14 days	About half hatched	
15 days	All hatched	
3 weeks	Larvae vigorous and would doubtless live indefinitely	Larvae larger and more active than control, evidently growing faster under like conditions

TABLE 2

Comparing the hybrids from *F. heteroclitus* ♀ × *F. diaphanus* ♂ with pure bred *F. heteroclitus*

TIME	PURE <i>F. HETEROCLITUS</i> (CONTROL)	<i>F. HETEROCLITUS</i> ♀ × <i>F. DIAPHANUS</i> ♂
2 hours	First cleavage	Same
4 hours	32 to 64-cell stages	Like control except that a small percentage of eggs are less advanced than any of the pure bred eggs, having 16 cells or less
20 hours	Advanced cleavage but no germ ring visible	Some embryos show the early steps in germ ring formation, others are less advanced than any of the control
52 hours	Blastopore closed or nearly so in all embryos, pale chromatophores appearing under hind-brain, a few red yolk chromatophores on yolk near head, no black yolk chromatophores	The normally developing embryos noticeably more advanced and more deeply pigmented than controls, <i>F. diaphanus</i> type of head chromatophores appearing on sides of mid-brain, a few black yolk chromatophores
3 days	Heart beginning to beat feebly in nearly all embryos, no vitelline circulation. A few black yolk chromatophores, and fewer head chromatophores	Heart beating quite strongly in a few embryos, the majority about as in control, a considerable percentage of abnormal, retarded embryos derived from the eggs that showed lagging cleavage at 4 hours
4 days	Vitelline and body circulation established and all types of chromatophores abundant	Almost identical with control in stage of development except the abnormal specimens, which from here on will be ignored as they never hatch; the normal embryos more heavily pigmented than control
5 days	Embryos nearly as advanced as those of <i>F. diaphanus</i> at 6 days	Very much like control; pigment characters given in detail later
1 week	Body shorter and stouter than in hybrid, otherwise little different except in details of pigmentation	Body rather more slender than control, a few more deeply pigmented than any of the controls
13 days	A few hatched	The majority hatched
14 days	All hatched	All hatched except abnormal specimens
3 weeks	Larvae vigorous	Larvae indistinguishable from control except in details of pigmentation

TABLE 3

Showing development of hybrids from F. diaphanus ♀ × F. majalis ♂; for control see first column of table 1

2 hours	First cleavage like control
6 hours	A smaller proportion of the more advanced stages of cleavage than in control
20 hours	Blastoderm much smaller than in control, germ ring scarcely distinguishable, no embryonic shield (fig. 9)
2 days	Germ ring one-third around the yolk, embryonic shield small and only slightly elevated from germ ring, embryonic axis not established (fig. 12)
3 days	Blastopore still open, in some cases germ ring is only three-fourths around yolk, optic vesicles flat with no optic cup, no lenses, no enlargement of hind-brain, no chromatophores
4 days	Embryo much less advanced than in control, no heart-beat, no body or yolk pigment (fig. 15)
5 days	Embryo about as advanced as control was at 4 days, chromatophores on head and yolk few as compared with control and all more delicately branched as in paternal species, no vitelline circulation but the majority of embryos have heart-beat established. Nearly half of the embryos appear at this time as if they were abnormal
1 week	The normal embryos have developed very rapidly since the establishment of vitelline circulation and are now nearly on a par with the control; many specimens, however, have failed to establish vitelline circulation and appear anemic and deformed
9 days	Even the more advanced embryos have lagged behind control; a number of abnormal embryos have died from anemia, the most pronounced differences between hybrids and controls have to do with pigmentation
12 days	None hatched
13 days	None hatched
14 days	A very few hatched
15 days	About a fourth hatched, larvae not very active
16 days	Nearly all of the normally developing embryos hatched; a few have died without hatching
3 weeks	Nearly all larvae dead, the few that have survived are not as large or as vigorous as the pure bred larvae of the same age, living under identical conditions

TABLE 4

Comparing hybrids from *F. majalis* ♀ × *F. diaphanus* ♂ with pure *F. majalis*

TIME	PURE <i>F. MAJALIS</i> (CONTROL)	<i>F. MAJALIS</i> ♀ × <i>F. DIAPHANUS</i> ♂
3 hours	In 2- and 4-cell stages	Same
6 hours	16 to 32 cells	Mostly 32-cell stages
22 hours	Blastoderm beginning to form faint germ ring	Blastoderm much broader than in control, germ ring well defined, embryonic shield beginning to appear as a thickening of germ ring
2 days	Germ ring about halfway around yolk, triangular embryonic shield, embryonic axis a thin line	Germ ring in more advanced embryos as much as two-thirds around yolk, embryonic axis more sharply defined than in control
3 days	Germ ring nearly around yolk but blastopore still widely open, optic vesicles flat without optic cups, no lenses, no mesoblastic somites	Blastopore closed, optic cups formed, lens flat, 4 to 7 somites
4 days	Embryos with many somites, well differentiated brain vesicles, no heart-beat, no circulation, no pigment	Embryos smaller but more advanced than control, no heart-beat or circulation, scattering slender black chromatophores on yolk, none on body
5 days	Embryo large, heart-beat and circulation well established, no chromatophores	Embryos about as advanced as control in general development, but much smaller in size, all show the paternal influence in pigmentation, since there are both black and red chromatophores on yolk and black chromatophores of the <i>F. diaphanus</i> type, or nearly so, on the sides of the hind-brain
1 week	Embryos very much larger than hybrids, a few slender black chromatophores on sides of mid- and hind-brains, slender black chromatophores on yolk, no red yolk chromatophores	Hybrids much smaller, eyes paler, head chromatophores almost pure paternal in type, black yolk chromatophores almost pure paternal, red yolk chromatophores intermediate in form and color between two parental types. Red chromatophores in three rows on side of body as in <i>F. diaphanus</i>

TABLE 4—Continued

TIME	PURE <i>F. MAJALIS</i> (CONTROL.)	<i>F. MAJALIS</i> ♀ × <i>F. DIAPHANUS</i> ♂
9 days	Head and body chromatophores all very finely branched and scattering, yolk chromatophores both red and black very slender	On the head the <i>F. diaphanus</i> type of chromatophore predominate, but there are always some of them with a tendency to branch like those of the maternal species; red yolk chromatophores much larger than in control and of an intermediate form and color. Body of embryos beginning to appear anemic on account of the interference with circulation produced by stretching of the heart, as in other <i>F. majalis</i> egg hybrids described elsewhere
18 days	Several hatched	Very little change since 9 days except that, a number of the weaker embryos have died
20 days	All hatched	Many dead, others very feeble. There has been very little advance since the first week of development except in the case of the chromatophores. None hatched in any experiment

b. Crosses between two genera

TABLE 5

Hybrids between F. diaphanus ♀ × *Cyprinodon variegatus* ♂

3 hours	About half of the eggs fertilized and in 2- and 4-cell stages, a few obviously polyspermic, others indistinguishable from control
5 hours	Advanced cleavage of regular character in the majority of developing eggs, but polyspermic eggs show great irregularity and considerable cytolysis
18 hours	All monospermic eggs normal in appearance but less advanced than control, the beginnings of cytolysis in a few blastodiscs
24 hours	All eggs show cytolysis of marginal cells of blastodisc, indicating that development has ceased and disintegration has set in. The limit of development in this cross seems to be the end of the cleavage period although a few embryos have begun to show the first indications of a germ ring, indicating that gastrulation or germ layer differentiation had begun but could progress no further

TABLE 6

Hybrids between Cyprinodon variegatus ♀ × F. diaphanus ♂

2 hours	About 75 per cent of eggs cleaving and 4- to 8-cell stages. Cleavage regular without any indications of polyspermy
6 hours	All fertilized eggs in advanced cleavage stages, like control but less advanced
20 hours	A few of the eggs that had cleaved are dead and partially disintegrated, but the majority have reached the end of the cleavage period and are beginning to show evidences of gastrulation. All are decidedly less advanced than control, in which all eggs are in germ ring stages
26 hours	Nearly half of the embryos that had reached the stage described above now show cytolysis, the others are still alive and in early germ ring stages
2 days	Only four embryos still alive. These have the germ ring one-third to one-half around the yolk, a triangular embryonic shield and a well-defined embryonic axis. Many embryos have died in early germ ring stages
3 days	Two embryos alive. These have blastopore nearly closed but show very little embryonic differentiation
4 days	Both embryos dead without having advanced beyond the condition noted on the previous day. It is possible for a few individuals of this cross to live practically through the period of gastrulation, but they are unable to enter upon embryonic differentiation. It is to be noted, however, that this cross succeeds much better than the reciprocal cross described in table 5

TABLE 7

Hybrids between F. heteroclitus ♀ × Cyprinodon ♂

2 hours	About 20 per cent of eggs cleaving. Polyspermy evident in nearly half of these eggs if one is to judge by the irregularity of blastomeres. The commonest irregularity consists of the production of two cells of very unequal size
5 hours	Nearly all that had cleaved are in advanced cleavage stages and appear to be quite normal. Evidently early irregularities have had little effect on the cell arrangements of this period
12 hours	Many eggs in advanced cleavage stages disintegrating. These are doubtless the polyspermic eggs. Others still normal in appearance
20 hours	All embryos dead, some evidently have but recently ceased to develop. These had succeeded in completing cleavage but show no signs of having entered upon gastrulation

TABLE 8

Hybrids between Cyprinodon ♀ × F. heterochilus ♂

2 hours	A large proportion of eggs cleaving and in 4- to 8-cell stages; no signs of polyspermy
5 hours	A great variety of cleavage stages, from 8 to more than 32 cells, for the most part regular or nearly so
20 hours	A few embryos have reached the end of the cleavage period and have started to form germ rings. The majority have begun to disintegrate at the end of the cleavage period
2 days	All those that were developing at 20 hours now dead, several having reached a condition in which the germ ring was about half around the yolk and a flat embryonic shield with no visible embryonic axis had been differentiated. This cross is more successful than the reciprocal cross, but less so than when the same eggs are fertilized by the sperm of <i>F. diaphanus</i>

TABLE 9

Hybrids between F. majalis ♀ × Cyprinodon ♂

3 hours	About 25 per cent of eggs cleaving and in 2- and 4-cell stages, many irregular and some evidently polyspermic
6 hours	Only a very few eggs alive, others show various degrees of cytolysis
10 hours	All dead and disintegrating after reaching advanced cleavage stages
	Note: Another cross of these two species gave even less success than that here indicated

TABLE 10

Hybrids between Cyprinodon ♀ × F. majalis ♂

2 hours	Nearly all eggs cleaving in an apparently perfectly normal manner.
4 hours	A wide range of cleavage stages ranging from 8 to 32 cells. Those that are much retarded look unhealthy
20 hours	All but two embryos dead in advanced cleavage stages. The two that are alive are in early germ ring stages, showing flat embryonic shield. Both are much less advanced than control
2 days	The two embryos that were alive at 20 hours are now dead without having made much progress since the last observation. This cross is more successful than the reciprocal, but less successful than either of the other <i>Cyprinodon</i> egg hybrids

3. SUMMARY AND DISCUSSION OF DATA SHOWN IN TABLES

a. Successful crosses

Out of twelve crosses possible between the four species dealt with in this paper four have been successful in the sense that development up to hatching took place and more or less viable larvae were produced.

The most successful crosses are those between *F. diaphanus* and *F. heteroclitus*, in spite of the fact that the former is a fresh water species and the latter a marine species. These two species cross reciprocally with equal facility, producing in each case some larvae that hatch earlier than those from pure bred eggs of the maternal species. These early larvae are also, in both reciprocal crosses, unusually vigorous and seem to excel the larvae of either parent species in viability and rapidity of growth. It is important to note here that *F. heteroclitus* develops somewhat more rapidly than *F. diaphanus*, but that the developmental rates of both reciprocal crosses are more rapid than those of the respective egg species, and that in the case of the *F. diaphanus* egg hybrids this is very marked. These facts are entirely out of accord with the observations and statements of Moenkhaus, who claims that the invariable effect of the foreign sperm in fish hybrids is to retard development, never to accelerate it. He considers this universal retardation to be the result of some injury to either the egg or sperm substances of the zygote, akin to the well known hemolytic effects observed in experiments dealing with the transfusion of foreign blood. The hemolysis parallel fails to apply, however, in these cases and in others where no retardation occurs but where a pronounced acceleration is evident to any one who takes the trouble to make carefully controlled studies of comparative rates of development of pure and hybrid strains. In these crosses in which early acceleration has been observed it is conceivable that we are dealing with effects akin to those rejuvenating or stimulating effects so often noted when diverse strains of the same stock are crossed. Certain new combinations of morphological and physiological characters are more readily produced and occur more rapidly

than those normal to either strain or species. I am inclined to interpret the speeding up of the developmental process in these hybrids as the result of the introduction by the sperm of a foreign enzyme, which produces abnormally rapid dissociations in the egg materials, and in this way hastens the processes of metabolism and development. Whatever be the chemical explanation of the acceleration the fact remains that at a very early period, certainly long before the end of cleavage, the hybrid eggs are developing more rapidly than the pure bred ones. This can be detected without the use of any refinement of method and should be obvious at a very much earlier period if the methods previously used by the writer (Newman '10) were employed. If anyone has abundant time and patience he might readily demonstrate an acceleration in the first cleavages, but the writer has foresworn any further attempts of this character as involving labor incommensurate with the reward involved.

Next in point of developmental success are the crosses between the eggs either of *F. diaphanus* or of *F. heteroclitus* and the sperm of *F. majalis*. Although the hybrids herewith treated develop successfully and produce a fair percentage of vigorous larvae they go much more slowly than the controls. It is also to be noted that the hybrids between *F. heteroclitus* ♀ × *F. majalis* ♂ are much more viable than those between *F. diaphanus* ♀ × *F. majalis* ♂.

An interesting commentary upon the generalization of Moenkhaus that foreign sperm always has a deleterious influence upon the development of the egg, resulting in retardation of development, is to be had from a study of the chart including figures 9 to 20. Here the developmental rates of eggs of *F. diaphanus*, fertilized by three species of *Fundulus* sperm are shown in three parallel vertical columns, the middle column showing the control (pure *F. diaphanus*) that on the left *F. diaphanus* eggs fertilized with *F. majalis* sperm, and that on the right eggs of *F. diaphanus* fertilized with *F. heteroclitus* sperm. It will be readily noted that the effects of the two types of foreign sperm are of an exactly opposite character, the one producing marked retardation and the other equally marked acceleration. No theory depending

on the idea that the effects of foreign sperm are always injurious, resulting in retardation, can explain both of these equally obvious results. Another point of rather general import is also illustrated by this chart. It often happens in cases where hybrid strains go faster or slower for the first few days of development, that, in so far as the general external evidences of degrees of development may be relied on as criteria, hybrid and pure strains reach a point at which they are for a short time on a par. This is well illustrated by the figures showing the three strains of *F. diaphanus* eggs at the end of one week (figs. 18, 19, 20). After this time acceleration or retardation is again evident, usually up to the period of hatching. I am unable at present to offer any suggestion as to the conditions underlying this apparent interruption in the developmental rhythms of these strains.

In all cases in which hybrids successfully weather the period of gastrulation and enter upon the period of embryo formation, further success seems to be conditioned by the ability or inability to establish nutritive relations with the yolk. In cases where hybrids differentiate a circulatory system but fail to complete the assimilation of yolk, the difficulty is evidently due to lack of balance between the rate of embryonic differentiation and that of yolk digestion, as will be shown later. Although in the hybrids under discussion (*F. diaphanus* ♀ × *F. majalis* ♂ and *F. diaphanus* ♂ × *F. majalis* ♀) a great many of the embryos become abnormal for these reasons, it must not be forgotten that in every experiment a large proportion of them surmount the difficulties of yolk assimilation and produce normal vigorous larvae, some of which, at least in the *F. heteroclitus* egg hybrids, are more viable and grow faster than either of the pure bred strains. It is difficult to imagine what factors underlie this wide range of success of individual hybrids of the same parentage. It has been suggested that the best results are obtained when both germinal products are at the optimum state of maturity, and that if either one or the other germ cell be at all under-ripe or stale the result is sub-optimal development of varying degrees depending on the degree of over- or under-ripeness of egg or sperm. A more nearly probable explanation, how-

ever, is based on an almost diametrically opposite assumption and is suggested by a series of experiments which I performed with the idea of testing the effects upon development of freshness and staleness of the germinal products. Similar results were obtained several times, but sometimes what seemed to be the same treatment gave different results. Eggs of *F. heteroclitus* were fertilized with the sperm of *F. majalis* which had been kept alive for about twenty minutes in 45 per cent sea-water, a concentration that greatly prolongs the life of these spermatozoa. A very small percentage of eggs cleaved, but these developed normally and showed scarcely any trace of the paternal influence either in rate of development or in details of inheritance; while the control strain, from the same batch of eggs fertilized at the same time by more sperm obtained fresh from the same male, showed the usual retardation in development and the typical hybrid characters described here and subsequently. It might then be concluded that, when the sperm is so stale that it is barely able to initiate development in the egg, it plays a rôle equivalent to that of the reagents that produce artificial parthenogenesis, but is unable to take part in the differentiation of hereditary characters. According to this view, *the most active sperms might have the most deleterious effects upon the egg materials of another species and give rise to serious incompatibilities whose result is more or less pronounced abnormality, and cessation of development; while the sperms that have lost some of their vigor are less likely to disturb the developmental rhythm of the egg and thus more likely to give normal embryos.*

b. Unsuccessful crosses

It will have been noted that, although the reciprocal crosses produce viable larvae, the hybrids between *F. majalis* ♀ and *F. diaphanus* ♂ and those between *F. majalis* ♀ × *F. heteroclitus* ♂ never produce larvae, since they never truly hatch. They may live for weeks within the egg membrane but gradually succumb to anemia. The difficulty is almost certainly one involving retarded or partial yolk assimilation, since in the final stages

of development there is a large external yolk sac full of undigested yolk. In fish the development of the pericardium, heart and vitelline circulation are intimately associated, and under normal conditions the yolk sac diminishes in size at such a rate that it, together with the pericardium and heart, are simultaneously drawn into the body cavity of the embryo shortly after hatching. In these abnormal hybrids, however, the differentiation of the heart and pericardium is at first accelerated, while yolk digestion goes more slowly than in pure bred embryos. Since the three structures are intimately associated, this failure of the yolk sac to diminish causes the pericardium to enlarge and stretches the heart into a long straight tube which beats feebly but carries, in later stages at least, no blood. Thus the supply of nutriment is cut off and growth ceases. Various organs continue to differentiate even without an external food supply, so that we may have an embryo developed which, though much smaller than the normal, has reached a stage of advancement equivalent to that seen in young larvae of pure bred strains. In last analysis the stoppage of development seems to be conditioned by a lack of coördination between two processes, that of the differentiation of the protoplasmic materials, which is accelerated by certain agents brought by the foreign sperm, and that of yolk assimilation which fails to progress as rapidly as in normal eggs. The result is that the embryo gets to the point when heart pericardium and yolk sac should be taken into the body cavity and is prevented from so doing by the large mass of undigested yolk. The yolk of *F. majalis* is optically denser and of different color from that of the other species and it may well be that a specific enzyme carried by the sperm of the same species is necessary for its complete dissociation and assimilation.

When I say that these abnormal embryos, burdened as they are with a permanent yolk sac, never hatch, I do not mean that they may not be shaken or dissected out of their membranes. When Bancroft claims that these embryos occasionally hatch I judge that he means that they may sometimes lose the egg membrane. Real hatching, however, is brought about by well marked violent struggling of the larva, and no such hatching struggles

have been observed in any of the numerous experiments dealing with *F. majalis* egg hybrids that have been under my observation during the last eight years. Moenkhaus, moreover, agrees with me that these hybrids never hatch.

c. Inter-generic crosses

It is possible to produce six crosses between *Cyprinodon* and the three species of *Fundulus*, as shown in tables 5 to 10. In no case is the cross successful in the sense that a larva or even an advanced embryo is produced. None of the hybrids go far enough to show specific characters, but all go through the cleavage period more or less normally and some develop through the period of gastrulation and begin to show embryonic differentiation.

The six crosses may be significantly arranged in the order of their success in development, as follows:

1. *Cyprinodon* ♀ × *F. diaphanus* ♂. A small percentage of the embryos go practically through the period of gastrulation and begin to show the rudiments of embryonic differentiation.

2. *Cyprinodon* ♀ × *F. heteroclitus* ♂. A large proportion of the eggs go through to the end of the period of cleavage in a nearly normal manner and a few advance to a stage in which the germ ring has covered about one-third of the yolk and a flat embryonic shield is developed. Here the stoppage occurs in the midst of the process of gastrulation and before the embryonic axis is established.

3. *Cyprinodon* ♀ × *F. majalis* ♂. A few embryos go through the cleavage period and show the beginnings and early steps in gastrulation, a well defined germ ring with flat embryonic shield being formed, as in figure 11.

4. *F. diaphanus* ♀ × *Cyprinodon* ♂. Many embryos go through the period of cleavage and a few of them form a germ ring but get no further, the most advanced condition being like that shown in figure 9.

5. *F. heteroclitus* ♀ × *Cyprinodon* ♂. Many embryos develop through the cleavage period but fail to begin gastrulation, as indicated by the absence of any trace of a germ ring.

6. *F. majalis* ♀ × *Cyprinodon* ♂. In two experiments performed at different times none of the eggs lived through the cleavage period but began to disintegrate during advanced cleavage stages.

It is clear that for some obscure reason all of the *Cyprinodon* egg hybrids succeed better than any of the three types of *Fundulus* egg hybrids. It may be that this difference in success between reciprocal crosses is due to differences in the sizes of eggs and that it will be found that greater success generally is associated with the smaller egg, less burdened with yolk. Whatever factors may be found to condition the differences in developmental success between reciprocal crosses we have at least the clear evidence of such differences and these facts militate against the idea that the degree of developmental success in teleost hybrids is simply a function of phylogenetic relationship. To make myself clear on this point let me review the facts concerning reciprocal crosses between *F. diaphanus* and *Cyprinodon*. The *Cyprinodon* egg hybrid succeeds in passing safely through the period of gastrulation and in making a considerable start at embryonic differentiation, while the *F. diaphanus* egg hybrid succeeds only in barely beginning gastrulation. There is a marked difference here in developmental success without any difference in phylogenetic relationship. There must then be some factor other than phylogenetic relationship at the basis of the difference between such reciprocal crosses.

It should be noted also that all of these six inter-generic crosses under discussion, though the parent species all belong to the same family, Poeciliidae, develop much less successfully than several crosses between species belonging to different orders, as for instance some of those cited by Moenkhaus. A cross between *Fundulus heteroclitus* of the order Haplomi and *Gasterosteus bispinosus* (stickleback) of the order Hemibranchiae, gives hybrids in which, although the germ ring is only one-third around the yolk, the "embryo is short and completely formed." Again a cross between *F. heteroclitus* of the order Haplomi and *Menidia notata* of the order Acanthocephali give hybrids in which the embryo is described as being sufficiently differentiated to show

optic vesicles. Numerous other cases of this sort are found in Moenkhaus's paper, while other crosses between these same pairs of orders give a much lower degree of success. Even Moenkhaus's own data, therefore, fail to support his claim as to the relation between kinship and developmental success.

d. General considerations concerning the influence of foreign sperm on the development and inheritance of teleost hybrids

After working in this field for a number of years, it now seems to me that we are dealing with two quite distinct types of phenomena, the first a matter primarily biochemical in character and the second a matter of genetics. When we are dealing with rates of development we are invading a field more closely related to chemistry than to heredity, but when we attend to the reappearance in offspring of the specific embryonic or larval characters of the parent species we are dealing with legitimate problems in heredity. In thus excluding from the realm of hereditary effects of foreign sperm those which have to do with mere rate of cleavage and later development, I realize that I am taking a position totally at variance with that to which I adhered with considerable tenacity for several years and which I maintained in earlier papers dealing with *Fundulus* hybrids. At the time when these papers were written the observations that most impressed me were those that had to do with the *rate of development* of hybrid embryos and the time of appearance of the various embryonic structures. In both reciprocal crosses between *F. heteroclitus* and *F. majalis* it happened that the rate of development of the hybrids was intermediate between that of the two parent species. This was true of the cleavage rate, rate of germing formation, time of appearance of first pigment and time of establishment of heart-beat and circulation. On this basis the conclusion was reached that, with regard to the great majority of characters studied, the blended rather than the alternative type of inheritance prevailed. Now, however, that I have many cases before me in which the rate of development of the hybrids bears no relation to that of the parents, I am convinced that

characters depending upon rate of development are not really matters of inheritance at all but matters primarily of physiological chemistry. For example, although *F. heteroclitus* develops at a higher rate of speed than *F. diaphanus*, both reciprocal crosses have a higher rate than the pure bred strains. There is no intermediate condition here and therefore no blended inheritance. Similarly, when we make reciprocal crosses between *Cyprinodon* and any species of *Fundulus* we find a marked retardation in developmental rate in both crosses, whereas if rate is a matter of inheritance we would expect the *Fundulus* egg hybrid to inherit from the *Cyprinodon* father a markedly accelerated rate. I am inclined to believe, on the basis of evidence now available, that it will be found generally true that *in crosses between very closely related species the rate of development will be accelerated, while those between distantly related species development will be retarded, but not necessarily in direct proportion to the heterogeneity of the cross.*

These are the considerations that have led me to modify my former views and to conclude that, *although foreign sperm may materially alter the rate of early development, it plays no rôle in the heredity of the organism until embryonic differentiation is well under way.*

INHERITANCE OF PIGMENT CHARACTERS IN FUNDULUS HYBRIDS

1. A STATEMENT CONCERNING PIGMENT CHARACTERS IN FUNDULUS SPECIES

The study of the inheritance of pigment characters in the various crosses is a complex and very difficult one, involving as it does a consideration of the development, time relations and distributions of several distinct types of chromatophores, each with its peculiar specific characters. In order to simplify the issue as far as possible I have endeavored in most cases to limit my descriptions to the definitive conditions of these cells as they appear in the young larvae or, in the cases of non-hatching hybrids, in embryos of a stage of differentiation equivalent to that of the pure bred larvae selected for study.

Pigment cells may be conveniently classed as 'body' and 'yolk' chromatophores. Four kinds concern us in this study: black body, black yolk, red body and red yolk. The red chromatophores differ specifically in form, size, color, number and distribution. They may readily be distinguished, even when the color with transmitted light may appear brown instead of red, by the fact that they have an opaque whitish appearance when seen by direct light. The black chromatophores range from dark brown to gray to black and differ specifically in the same respects as the red cells.

Bancroft ('12) has added very materially to our knowledge of the inheritance of pigment characters in teleost hybrids. Using the same two species of *Fundulus* that furnished the basis of my previous experiments he has carried his analysis of this phase of heredity much further than I did. In my new experiments I have had occasion to repeat much of this work of Bancroft's and find little to criticize concerning his observations. If I were to attempt a general criticism of the work I would be inclined to ask whether he has not allowed his tendency to find Mendelian dominance in all pigment characters to obscure his vision for the many evidences of incomplete dominance or blending. If one is on the lookout for cases of dominance and is not too particular about its purity one can find an abundance of examples of it; but if one is on the alert for intermediate conditions or mosaics of maternal and paternal units, he can find abundant examples of them in every hybrid. Whether or not any particular significance attaches to the more or less pure dominance of unit characters in F_1 hybrids between Linnean species, is an open question that we need hardly discuss here. Doubtless, were these hybrids fertile and capable of intercrossing, the parental characters would segregate out in the F_2 generation of hybrids. Since no one has been able to get a second generation of teleost hybrids and since there is already a considerable literature on the inheritance of F_1 hybrids between teleosts, it seems worth while to try to clear up the situation and to remove existing discrepancies as far as possible. With this idea in view I have performed these additional breeding experiments and offer the following considerably

wider range of data on pigmentation derived from four additional *Fundulus* crosses.

Bancroft's table on page 156 gives in brief form a characterization of the pigment differences found by him in the embryos and larvae of pure bred *F. heteroclitus* and *F. majalis* together with their reciprocal crosses. For convenience I shall use this table as a starting point, modifying it where it seems to me to be necessary and adding to it where it does not provide for new characters seen in the new hybrid types. For the sake of brevity the often repeated word 'chromatophore' is abbreviated to 'c.'

2. TABULATION OF DATA ON PIGMENT INHERITANCE

TABLE 11

Showing pigment characters in Fundulus species, based on Bancroft's table 1, emended and expanded

NO.	F. HETEROCLITUS	F. MAJALIS	F. DIAPHANUS
1	Red yolk chromatophores large and abundant, much branched and bright red (fig. 21)	Red yolk c. much fewer than in F. het., very small and inconspicuous with a few fine branches, bright red color (fig. 32)	Red yolk c. large and abundant, much like F. het. except that color is red-brown (fig. 30)
2	Black yolk c. large and of a general polygonal shape, processes usually wanting	Black yolk c. smaller and with more and larger branches than in F. het.	Black yolk c. larger and darker than F. het., also rounder in form and with many short radiating branches
3	First crop of head c. black and few in number, appearing about four days after fertilization; an interval before second crop	First crop head c. absent	First crop of head c. brown, numerous, appearing about three days after fertilization; no interval before second crop, as in F. het.
4	Lateral line with one continuous line of red c. at or before hatching; an occasional red c. far out of line	Lateral line without red c.	Three rows of red-brown c. on sides of body and tail, the middle one on the lateral line
5	Lateral line with none or but few black c. upon hatching	Lateral line with about 50 black c. upon hatching	Lateral line with about 30 black c., alternating with red-brown c., upon hatching
6	When yolk c. first appear there are as many on side opposite embryo as on embryonic side	Yolk c. first appear at sides of embryo and are entirely absent from opposite side	Yolk c. first appear on side opposite embryo and are larger and more numerous there than elsewhere
7	First yolk c. appear before any head c., at about 3 days	First yolk c. appear at about 5 days	First yolk c. appear after first head c., about 4 days after fertilization
8	Red c. on head	No red c. on head	Red-brown c. on head
9	Much fusion of black c. on head and yolk (fig. 22)	No fusion of any c. (fig. 23)	No fusion of any c. (fig. 21)

TABLE 12

Showing pigment characters in hybrids between F. diaphanus and F. heteroclitus

NO.	F. DIAPHANUS ♀ × F. HETEROCLITUS ♂	F. HETEROCLITUS ♀ × F. DIAPHANUS ♂
1	Red yolk c. intermediate in color between the maternal red-brown and the paternal brick red (fig. 33)	Same as reciprocal cross, but a little smaller on the average (fig. 34)
2	Black yolk c. like F. diaphanus in size and branching, but show a tendency to fuse as in F. heteroclitus	Same as reciprocal cross except that the type of c. is again almost pure F. diaphanus and there is scarcely a trace of fusion
3	Both maternal and paternal types of black head c. present; the maternal type appears first at about 3 days, followed by the paternal type in about 4 days (the first crop of Bancroft); each type has the characteristics of the pure species (fig. 24)	All black head c. of the F. diaphanus type, but there is much fusion as in F. heteroclitus (fig. 25)
4	Three rows of red-brown c. on sides of body, intermediate in color between those of two species	Same as reciprocal cross except that there are fewer c. to the row, the body being shorter
5	Black yolk c. larger and darker on side opposite embryo than elsewhere, as in maternal species	Black yolk c. less numerous on side opposite embryo than elsewhere; this is not like either parent
6	Lateral line with number of black c. intermediate between two species	Same as reciprocal cross
7	First yolk c. appear as early as in F. heteroclitus; time not intermediate	Same as reciprocal cross
8	Red c. of head intermediate in color between two species	Same as reciprocal cross
9	Fusion in the F. heteroclitus type of black c., but the F. diaphanus type isolated (fig. 24)	All black c. of the F. diaphanus type and fused extensively (fig. 25)

TABLE 13

Showing pigment characters in hybrids between F. diaphanus and F. majalis

NO.	F. DIAPHANUS ♀ × F. MAJALIS ♂	F. MAJALIS ♀ × F. DIAPHANUS ♂
1	Red yolk c. distinctly intermediate between the two parental types, in color, size and degree of branching (fig. 35)	Same as reciprocal cross, except that the paternal tendency to branch is stronger in (fig. 36)
2	Black yolk c. much smaller and more branched than in F. diaphanus, but larger and less branched than in F. majalis; a striking case of blended inheritance	Black yolk c. nearly as large and conspicuous as in F. diaphanus, but there is a marked tendency to give off long processes as in F. majalis
3	Black head c. practically pure F. diaphanus in form and size, but they appear later than in maternal species. This cannot be explained as the result of two crops (fig. 26)	Black head c. intermediate in type between those of two parent species (fig. 27); time of appearance also strictly intermediate
4	Lateral line with three rows of red-brown c. as in F. diaphanus, color intermediate	Same as reciprocal cross; here is a case of almost pure dominance of a paternal character
5	Lateral line with number of black c. intermediate between two species	Same as reciprocal cross
6	Black yolk c. less numerous on side away from embryo than in F. diaphanus; character intermediate	Same as reciprocal cross
7	Time of appearance of first yolk c. strictly intermediate	Same as reciprocal cross
8	Red c. of head and lips as numerous or nearly so as in F. diaphanus, but color is brighter than in maternal species	Red c. of head and lips seemingly larger and more conspicuous than in dominant parent species, color intermediate; case of hyperdominance
9	No fusion of c., since fusion is not a character of either parent species	Same as reciprocal cross

Pigment characters of hybrids between F. heteroclitus and F. majalis

It should not be necessary to repeat the facts concerning these crosses since they form the subject-matter of Bancroft's paper. An examination of his table 1 will reveal the facts as they appeared to him. A reëxamination of the same data, however, has convinced me that there is much more blending or intermediate inheritance and less pure dominance than Bancroft leads us to expect. He lists the following characters which he believes to be dominant:

1. "The character—presence of many large red yolk chromatophores (F. heteroclitus condition) is dominant over the character—presence of few small yolk chromatophores (F. majalis character)." Yet in the table and elsewhere he states that in the hybrids these elements are fewer, smaller and less branched than in the dominant parent.

2. "The size and shape of the black yolk chromatophores of F. heteroclitus is dominant over the size and shape of these same cells in F. majalis." Yet in his table we learn that these cells are smaller and have many minute processes, showing that the F. majalis influence is evident in the matter of size and shape.

3. "The presence of a first crop of head chromatophores appearing before the majority of the head chromatophores (F. heteroclitus condition) is dominant over absence of this crop of head chromatophores (F. majalis condition)." I have been unable to convince myself of the validity of this character since in many cases I have observed that the first crop consists of one chromatophore, that two or more followed after a considerable interval, and that the others came on gradually so that I have been unable to decide what constituted the first crop. All that I have been able to say for certain is that the first few chromatophores seem to be somewhat larger and to take longer to develop than the subsequent ones. A similar situation exists in F. diaphanus and here there is no distinction between the earlier and later chromatophores. I still believe that the time of appearance of the first chromatophores in the hybrids is strictly an intermediate character, and as such not an example of dominance.

4. "The presence of red chromatophores along the lateral line at hatching time or shortly before it (*F. heteroclitus* condition) is dominant over the absence of red chromatophores at the same time (*F. majalis* condition)." This is, beyond question, a case of dominance based on the presence of a positive character in one species and its absence in another. Bancroft fails, however, to mention that in the hybrids these red lateral line chromatophores are smaller and fewer in number than in pure *F. heteroclitus*.

To this list of Bancroft's I might add what seem to me to be two other characters as dominant as those just given. These are:

5. The character—red chromatophores on the head (*F. heteroclitus* character) dominant over the absence of red chromatophores on the head (*F. majalis* character). These red chromatophores are, however, fewer in number and smaller in the hybrids than in pure *F. heteroclitus*.

6. The character—fusion of black chromatophores (*F. heteroclitus* condition) is dominant over absence of fusion (*F. majalis* condition). In the hybrids, however, fusion is never so complete as in pure *F. heteroclitus*.

It is then apparent that no pure dominance is to be found in these hybrids and that partial dominance occurs only in such cases as those cited, in which a character is present in one species and absent in another.

3. SUMMARY AND DISCUSSION OF DATA ON PIGMENT INHERITANCE

1. When a character is present in one species and entirely absent in the other, the hybrids between the two species exhibit dominance of a more or less perfect type. Examples of this type of inheritance are as follows:

a. The character—one row of lateral line red chromatophores of *F. heteroclitus* is dominant over the absence of lateral line chromatophores in *F. majalis*, but the individual chromatophores are smaller and less complexly branched in the hybrids than in *F. heteroclitus*.

b. The character—three rows of lateral red body chromatophores of *F. diaphanus* is dominant over the single row of *F. heteroclitus* and the total absence of red body chromatophores of *F. majalis*. But in hybrid between *F. majalis* ♀ × *F. diaphanus* ♂ these chromatophores though brighter in color than in *F. diaphanus* are darker than in *F. majalis* and are larger and more conspicuous than in either parent species. This is a case of what in a previous paper I called hyperdominance.

c. The character—red chromatophores of forebrain and lips of *F. diaphanus* and *F. heteroclitus* is dominant over its absence in *F. majalis*, but in all crosses in which *F. diaphanus* is used there is a blending between brown color of *F. diaphanus* and the bright red of the other two species.

d. The character—fusion of head chromatophores of *F. heteroclitus* is dominant over its absence in the other species, but the degree and extent of the fusion in the hybrids is always less than in the dominant parent.

2. When a character occurs in both parent species but differs in the degree in which its various attributes express themselves, two alternative modes of inheritance are seen in the hybrids; either there appears a mosaic of the two types, each nearly pure in detail, or each of the units shows an intermediate condition as to the degree in which the various specific attributes find expression. Examples of both of these modes of inheritance are cited as follows:

a. In hybrids between *F. diaphanus* and *F. heteroclitus* both specific types of black head chromatophore appear to be dominant and occur side by side—a case of *mosiac or particulate inheritance*. Each type behaves throughout development like the homologous parental type, for example the *F. heteroclitus* type fuses with its neighbors to form small masses, while the *F. diaphanus* type remain as isolated cells.

b. In crosses between *F. heteroclitus* and *F. majalis* there is a blending with respect to the details of both red and black chromatophores wherever these occur. In the hybrids of both reciprocal crosses the individual chromatophores are smaller and

more finely branched than in *F. heteroclitus* and larger and less finely branched than in *F. majalis*. This is typical *blended inheritance*.

c. In crosses between *F. diaphanus* and either of the other species the color of the red chromatophores of the hybrids is distinctly *intermediate* between that of the two parental species.

3. When numbers of integral variates are concerned the hybrids exhibit a number intermediate between those of the two parents; or when one parent has many of a given type of integral variate and the other has none the number in the hybrids is less than that in the parent showing the characters in question. The following examples illustrate this:

a. In both reciprocal crosses between *F. heteroclitus* and *F. majalis* the hybrids show an intermediate condition between the *F. heteroclitus* character, few or no lateral line black chromatophores, and the *F. majalis* character, many such cells.

b. In the cross between *F. majalis* ♀ × *F. diaphanus* ♂ there are fewer lateral red chromatophores to the row than in *F. diaphanus*, although there are no lateral red chromatophores in *F. majalis*.

c. In hybrids between *F. diaphanus* and *F. heteroclitus* the hybrids have fewer black head chromatophores of the *F. diaphanus* type than has pure *F. diaphanus* and fewer black head chromatophores of the *F. heteroclitus* type than has the pure *F. heteroclitus*, yet the combined number of the two types of chromatophores is, as nearly as I can determine, intermediate between the total numbers of the two parent species.

When, therefore, we analyze one set of inherited characters such as the pigment characters that we have just dealt with, we find practically all of the well-known modes of inheritance; that characterized by *dominance* or even *hyperdominance*; that commonly called *blended* or *intermediate* inheritance; that usually called *mosaic* or *particulate* inheritance; and that which is peculiar to integral variates and has sometimes been classed as a phase of intermediate or blended inheritance, but which seems to me to be *sui generis*.

It is still an open question whether all of these types could successfully be reduced to an orthodox Mendelian basis, were the unit factors involved all analyzed out. Personally I would not enjoy the prospect of untangling these ultimate inheritance units from the conglomeration that exists in the hybrids of the F_1 generation. If such an analysis were possible at all it could be made only from data derived from F_2 hybrids. Any attempt to proceed very far with such an analysis upon the F_1 hybrids would seem to be pushing the factor hypothesis too hard, without being of any value to the subject of genetics, for the principle of *dominance* is at best of minor significance as compared with the principle of *segregation*. In the F_1 generation of hybrids we may readily have pure dominance, blends, intermediates, mosaics or particulate mixtures without doing violence to the essentials of Mendelian inheritance, for the original unit factors, whether pure dominants, blends or mixtures, segregate out in the pure state in the next generation of hybrids, thus satisfying all of the demands of the theory.

This then is my conclusion: that the F_1 generation of hybrids affords unfavorable material for the study of Mendelian inheritance and that unless we can interbreed the F_1 hybrids we can make little progress in the analysis of the inheritance factors. Yet certain facts of much importance from the standpoint of developmental physiology may be determined by means of teleost hybrid material, some of which we have discussed and shall more fully discuss in this paper. I still feel as I did when I wrote my earlier papers that for the purpose of Mendelian breeding work teleosts are unfruitful material, but that they throw a great deal of light on the process of heredity. In this respect teleost material is no better and no worse than echinoderm material, which has been so extensively exploited.

GENERAL SUMMARY OF CONCLUSIONS

1. The nature of the influence of the spermatozoön on cleavage and development

The new evidence here offered tends to support the position that the rate of cleavage is largely a function of egg size and egg constitution and is inherited only in this restricted sense, just as certain factors of the environment may be said to be inherited, because the organism finds them ready made for its use. Foreign sperm, when introduced into the egg, may accelerate or retard cleavage or later development, but in so doing is not exercising an hereditary function, for there are cases in which the sperm of a more rapidly developing species fetards development and others in which the sperm of a more slowly developing, but closely related species, hastens development. This is surely not heredity. I would like to suggest that the influence exerted by foreign sperm upon the rate of development is chemical and mechanical in nature and that it is only when the sperm components of the zygote begin to coöperate with the egg components in bringing about the differentiation of specific characters, that the sperm begins to play its rôle as a factor in heredity. It is doubtless during the process of gastrulation that the first steps in differentiation take place and it is very interesting to note that in so many heterogenic crosses the developmental stoppages occur at the onset of or during the process of gastrulation. The conclusion would seem to be obvious then that any teleost spermatozoön may play a rôle in cleavage equivalent to that of agents that are successful in artificial parthenogenesis, but that only certain special kinds of sperm, that can successfully coöperate with the egg nucleus in its hereditary activities, are capable of working out a complete ontogeny.

2. *Harmful versus beneficial effects of foreign sperm*

The effects of foreign sperm are not always deleterious nor is it true that cross fertilization always results in a retardation or interruption of early development, as Moenkhaus maintains. In fact the sperm of a closely related species may, and actually does in three out of six *Fundulus* crosses, definitely accelerate development from the very beginning, and produces a hybrid larva more viable and capable of growing faster than those of either parent species. The idea of Moenkhaus then, that foreign sperm plays a rôle equivalent to that of foreign blood in transfusion experiments, is out of accord with these facts, for there is nothing equivalent to the injurious hemolytic effects of foreign blood in crosses where the sperms of closely related species are used. On the contrary, an effect is produced akin to the well-known stimulating effects of crossbreeding strains in practical animal husbandry.

3. *Phylogenetic relationship of parent species and developmental success of hybrids*

The idea expressed by Moenkhaus that success in the development of teleost crosses is a function of the phylogenetic relationship of the parent species breaks down in view of two sets of facts:

a. There is a marked difference between the developmental success of reciprocal hybrids. The parents are the same in both, hence factors other than that of phylogenetic relationship must underlie this different degree of success in development.

b. The data given in this paper, and by Moenkhaus himself, prove that success in development of hybrids produced by crossing different orders of teleosts is not seldom greater than that attained by hybrids between different families of the same order, or even between different genera of the same family.

Kammerer endeavors to show that success in crossing depends on similarity of habitat, but in these experiments of mine a fresh water and a marine species give the most normal development, while two marine species give unsuccessful crosses. The

problem as to the factors involved in the relative developmental success of reciprocal crosses, and of hybrids between various species, is unsolved.

4. Modes of inheritance in hybrids and the Mendelian hypotheses of dominance and segregation

It has been shown that structural and physiological characters in the F_1 generation of teleost hybrids show exclusive, blended and particulate inheritance. There is seldom if ever pure dominance and there is frequently an almost perfect intermediate condition between parental types, especially in the case of pigment characters. In some cases there is a sort of regional dominance of one parental type in one area and a similar regional dominance of the other parental type in another area; or else the two types may exist side by side in quite intimate contact with each other. These mosaics and mixtures are typical examples of what we have come to call particulate inheritance. In the case of integral variates the hybrids exhibit numbers of variates intermediate between those of the parents. So we find practically all known types of inheritance in connection with pigment characters alone, and would also find a similar condition for any other group of characters studied equally intensively. The discovery of the prevalence of intermediates and blends in the F_1 generation does no violence to the fundamental principles of Mendelian inheritance, for it is practically certain that these characters that seem to blend in the first generation of hybrids would segregate out in typical fashion in subsequent hybrid generations, were it possible to interbreed the F_1 hybrids. We have nothing to offer, therefore, that need be interpreted as out of accord with recent Mendelian work, although previous work dealing with the same kind of data has been cited not infrequently as furnishing examples of non-Mendelian inheritance.

5. Inheritance in reciprocal crosses

It must be obvious to the reader that in these and other teleost crosses reciprocal hybrids differ materially in many respects. It has been shown repeatedly that in the majority of cases where in the hybrid a character appears as a blend between that of the two parents, it approximates more closely the maternal than the paternal condition. There are, however, exceptions in which the paternal influence predominates. Again we have cases in which in one reciprocal cross a given character will be almost entirely like that of one parent, whereas in the other reciprocal cross we may have a mosaic or a blend of the two parental conditions. There are also cases in which the male influence predominates in both reciprocal crosses, but is less pronounced in one cross than in the other. The facts do not admit of being classed in the category of sex-linked characters, for all hybrids of a strain, which presumably consists of equal numbers of males and females, show the same conditions. Nor is the inequality of reciprocal hybrids to be explained by assuming that there may be a partial elimination of paternal chromatin, as was the case in Baltzer's echinoderm hybrids, for we have abundant evidence that in all of these *Fundulus* crosses the paternal chromatin functions normally. There is evidently a problem here the solution of which demands a much larger range of data than has as yet been obtained. I am for my own amusement positing a number of tentative hypotheses that may or may not stand the test of experiment, but shall not risk at present a premature display of a tendency to jump to conclusions. As it stands the problems involved in the differences between reciprocal crosses are unsolved.

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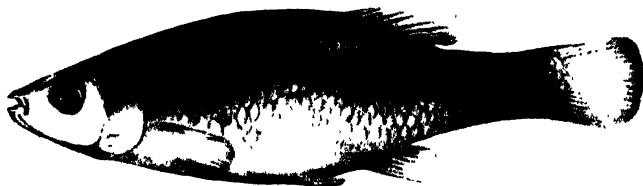
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PLATE 1

EXPLANATION OF FIGURES

1 to 8 represent the four species of fish used in the present experiment. Both males and females are shown life sized.

- 1 *Fundulus heteroclitus* adult female.
- 2 *Fundulus heteroclitus* adult male.
- 3 *Fundulus majalis* adult female.
- 4 *Fundulus majalis* adult male.



1



2



3



4

PLATE 2

EXPLANATION OF FIGURES

- 5 *Fundulus diaphanus* adult female.
- 6 *Fundulus diaphanus* adult male.
- 7 *Cyprinodon variegatus* adult female.
- 8 *Cyprinodon variegatus* adult male.



5



6



7



8

PLATE 3

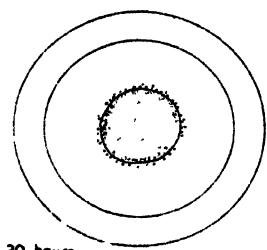
EXPLANATION OF FIGURES

9 to 20 form a developmental chart, showing the comparative rates of development of embryos of pure *F. diaphanus* (middle column) and the two types of *F. diaphanus* egg hybrids (that from *F. majalis* sperm on the left and that from *F. heteroclitus* sperm on the right). Note that *F. majalis* sperm retards early development and that *F. heteroclitus* sperm as markedly accelerates it.

F. diaphanus ♀
×
F. majalis ♂

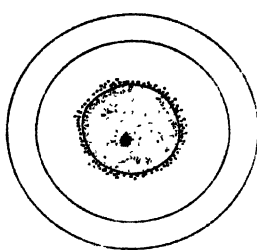
F. diaphanus ♀
×
F. diaphanus ♂

F. diaphanus ♀
×
F. heteroclitus ♂

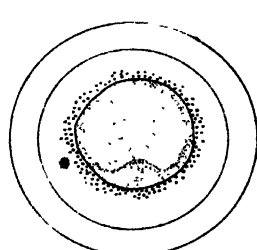


20 hours

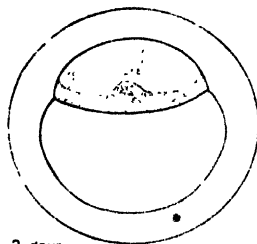
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10

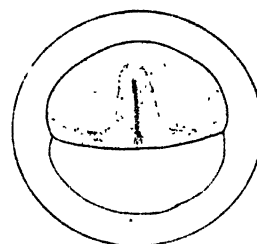


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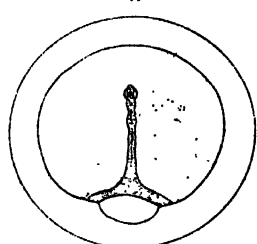


2 days

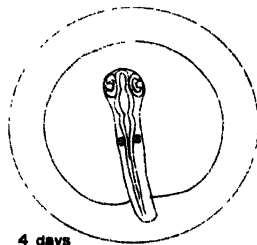
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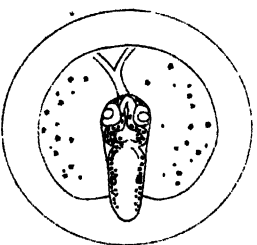


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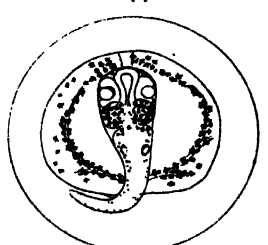


4 days

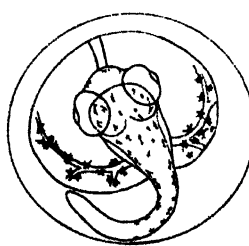
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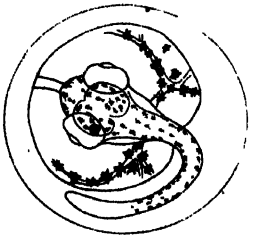


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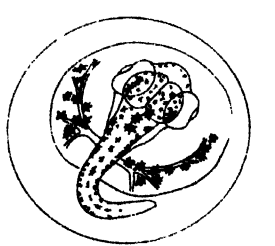


7 days

18



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PLATE 4

EXPLANATION OF FIGURES

21 to 29 Show the various types of black head chromatophores found in young larvae, at hatching or at a period equivalent to this in non-hatching embryos. All camera drawings under same magnification ($\times 100$).

21 The isolated stellate type of *F. diaphanus*.

22 The polygonal, unbranched, fusing type of *F. heteroclitus*.

23 The radiate type with tooth-like branches on the rays, characteristic of the hatched larvae of *F. majalis*.

24 A typical group of head chromatophores in the hybrid larva from egg of *F. diaphanus* and sperm of *F. heteroclitus*. Note the mosaic of isolated, stellate *F. diaphanus* cells and the fused groups of polygonal *F. heteroclitus* cells.

25 A typical group of head chromatophores of the reciprocal hybrid larva, from *F. heteroclitus* egg and *F. diaphanus* sperm. Here we find only the stellate cells of *F. diaphanus*, but they show the *F. heteroclitus* character of fusion.

26 A typical group of head chromatophores from the hybrid larva from *F. majalis* egg and *F. diaphanus* sperm. The paternal type is almost entirely dominant.

27 A typical group of head chromatophores from the reciprocal hybrid from *F. diaphanus* egg and *F. majalis* sperm. The cells are intermediate in size and form between those of the parent species.

28 A typical group of head chromatophores in hybrid larva from *F. heteroclitus* egg and *F. majalis* sperm. The *F. heteroclitus* polygonal fused type predominate, but in certain regions a modified *F. majalis* type of cell is fairly abundant.

29 Group of head chromatophores in hybrid from *F. majalis* egg and *F. heteroclitus* sperm, showing dominance of the paternal type.

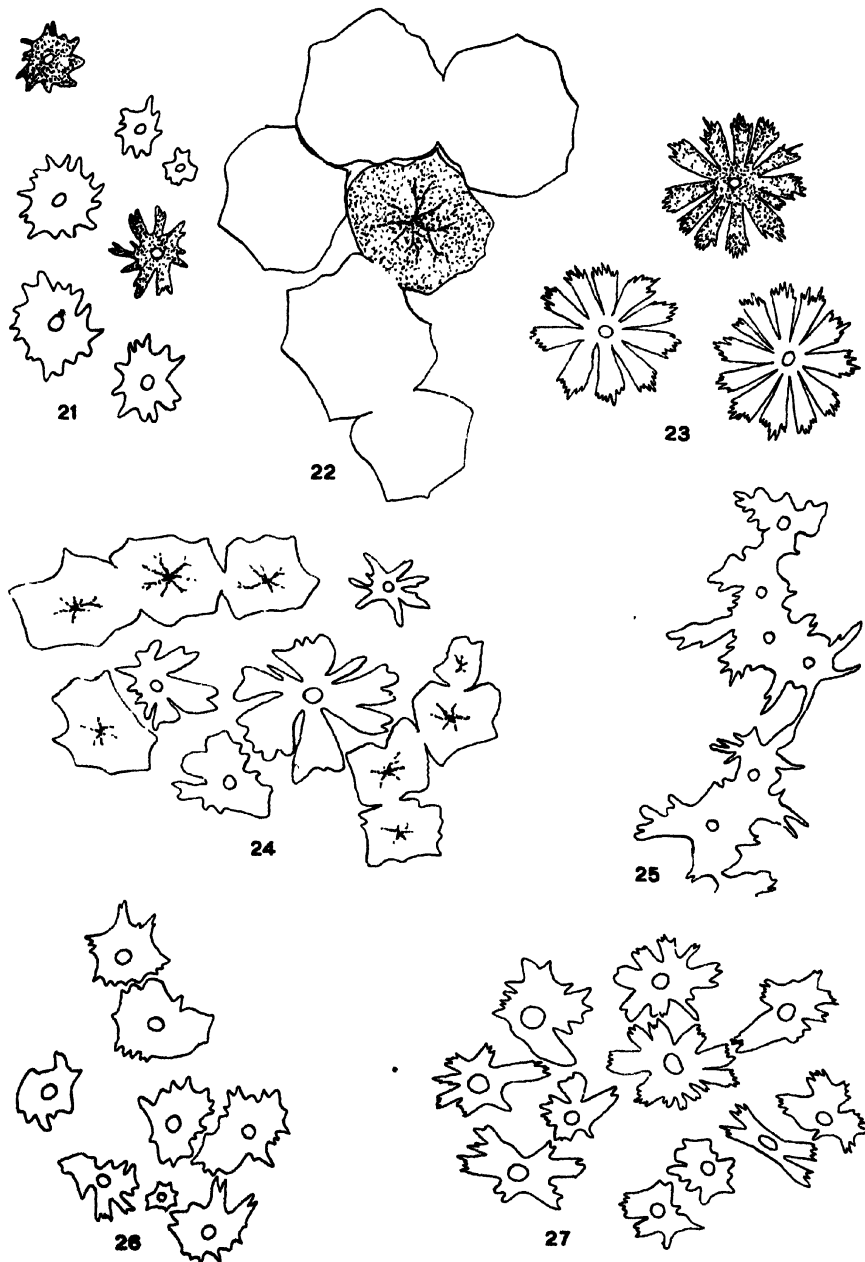


PLATE 5

EXPLANATION OF FIGURES

30 to 38 Represent approximately the form and color of the red yolk chromatophores in pure and hybrid strains.

30 Red-brown cell of pure *F. diaphanus*, showing profuse branching.

31 Brick red cell of pure *F. heteroclitus*, showing large size and many branches.

32 Brick red, but very small inconspicuous cell of *F. majalis*, showing hair-like branches.

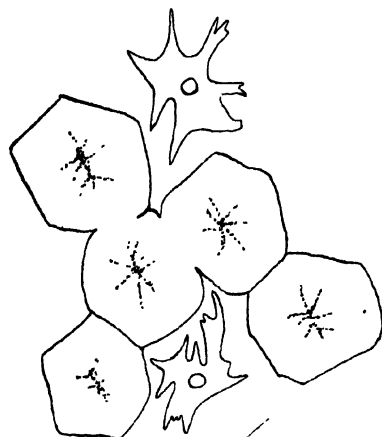
33 Type of red chromatophore found in hybrid larvae from *F. diaphanus* egg and *F. heteroclitus* sperm. These cells are less branched than in *F. diaphanus* and are intermediate in color between red-brown and brick red.

34 Type of red yolk chromatophore found in hybrids from *F. diaphanus* sperm and *F. heteroclitus* egg. Branches are shorter than in reciprocal cross; color same as reciprocal cross.

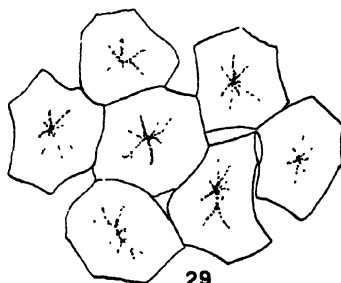
35 Type of red chromatophore in hybrids from *F. diaphanus* egg and *F. majalis* sperm. Strictly intermediate in every particular.

36 Type of red chromatophore in hybrids from *F. majalis* egg and *F. diaphanus* sperm. Like reciprocal cross except that cells are more branched.

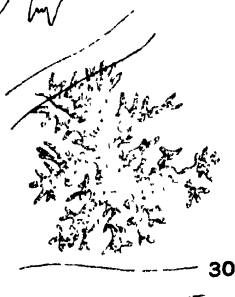
37 and 38 Types of red chromatophore in the two reciprocal crosses between *F. heteroclitus* and *F. majalis*. Fig. 37 the *F. heteroclitus* egg hybrid, figure 38 *F. majalis* egg hybrid. In both cases the intermediate condition is evident.



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29



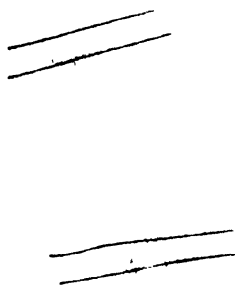
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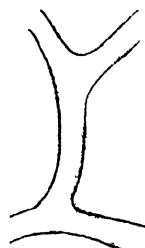
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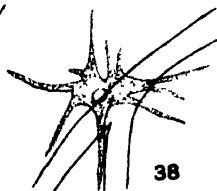
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38

THE BEHAVIOR OF THE CHROMATIN IN HYBRIDS BETWEEN FUNDULUS AND CTENOLABRUS

MARGARET MORRIS

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THIRTY-SIX FIGURES (FIVE PLATES)

The following investigation was begun in the summer of 1911 at the suggestion of Dr. Kellicott of Goucher College, and the material was collected at Woods Hole in the summers of 1912 and 1913. I am indebted not only to Dr. Kellicott for the suggestion, but also to the members of the Zoological Faculty of Yale University for the privileges extended to me in the Yale laboratories, and for their advice and encouragement while I was following out the problem.

Fundulus heteroclitus and the cunner, *Ctenolabrus adspersus*, are common in the waters about Woods Hole. The spawning season for both kinds of fish is approximately the same, extending from the last of May till the first of July. The eggs are easy to get from both species, and the sperm of *Ctenolabrus* can be easily expressed. The male *Fundulus* is more difficult to strip and it is best to tease out the testis in a watch-glass, add a few drops of water, and use this fluid to fertilize the eggs. The *Fundulus* eggs can be obtained at any time of day, and from females that have been some days in the laboratory; but the *Ctenolabrus* eggs are not good if taken from a female that has been in the aquarium over night. As the eggs do not ripen till late in the afternoon, it is best to have a fresh supply of *Ctenolabrus* brought in not earlier than 3 p.m. A few males will probable live over night, and their milt can be used for *Fundulus* eggs the next morning.

The usual precautions were taken to prevent normal fertilization of the eggs; that is, fertilization by the sperm of their

own species. The pipettes were not only carefully washed, but were marked besides, so that the one used for *Fundulus* sperm was never used for unfertilized or hybrid *Fundulus* eggs. Dishes were carefully washed and dried, and a control lot of unfertilized eggs from the same lot as the hybrids was always kept. No eggs were found in any of these control lots that had divided.

The eggs of *Fundulus* have a diameter of about 2 mm., while those of *Ctenolabrus* are about 1 mm. in diameter. Both kinds were handled as follows: they were expressed into a finger-bowl containing a small amount of water. A number were taken out for two controls, one of which was normally fertilized and the other left unfertilized. The rest of the eggs were fertilized with the foreign sperm, and more water was added to all three lots to keep them fresh.

The eggs were preserved in series at varying intervals, the fluids used being Gilson's, Bouin's, and Perenyi's for the *Fundulus* eggs and Flemming's strong mixture and Perenyi's fluid for the *Ctenolabrus* eggs. The method of embedding and sectioning used by Moenkhaus on *Fundulus* eggs worked very well for those preserved in Bouin's fluid. After the tough outer membrane had been removed, the eggs were embedded with the blastodisc uppermost, and sections 8 to 10 μ in thickness were cut horizontally through the blastodisc. The eggs preserved in Gilson's and Perenyi's fluids were differently treated, as a touch of the needle removes the blastodisc from the yolk, and it is best to embed the former alone. The *Ctenolabrus* eggs are more troublesome. They are too small to be easily embedded in a definite position, so the sections must be cut at random. Moreover, those preserved in Flemming's mixture are so dark that it is necessary to bleach them. I used Mayer's method, bleaching the sections on the slide, but found that sections so treated did not take the stain well. The stains used were Heidenhain's hematoxylin and iron alum, and Ehrlich's hematoxylin counterstained with Congo red. The latter is more satisfactory, as there are granules in the cytoplasm of the *Fundulus* egg which are stained by the iron hematoxylin method and may be confused with small chro-

mosomes. With Ehrlich's hematoxylin, however, no mistake can be made, as it is only the chromatic material that takes the deep purple stain, all the rest of the egg being stained red.

No records were made of the percentage of eggs dividing in the hybrid cultures, so that only the most general statements can be made on this point. The *Fundulus* eggs are fertilized readily with the *Ctenolabrus* sperm and it is estimated that about half of those in a hybrid culture go through cleavage. Division was much rarer in hybrid *Ctenolabrus* eggs, not more than 10 per cent of them being fertilized. The head of the *Fundulus* sperm is considerably larger than that of the *Ctenolabrus* sperm (figs. 35 and 36) and this may account for the greater ease with which the $F \varphi \times C \sigma$ cross is made.

Polyspermy is apparently rare. Only one tetraster was found in the preserved *Fundulus* material, and one case is recorded of a 4-cell stage in a culture in which the other eggs were in the 2-cell stage. This was probably a dispermic egg, dividing at once into four cells. There was no evidence of polyspermy in the *Ctenolabrus* eggs, but all the evidence from these eggs is poor, and polyspermy may possibly occur in them.

In neither cross did I succeed in raising the hybrids to a late stage of development. Loeb has raised hybrids from the same two species of fish, but it is necessary to put only three or four eggs into a single dish, and take particular care to change the water frequently. Even then the proportion of late stages is small, so that to have satisfactory results one must fertilize large numbers of eggs daily. With the limited space at my command, I was unable to use this method. The conditions under which I kept the eggs were perfectly satisfactory for the normally fertilized ones, but not for the less hardy hybrids. The *Fundulus* eggs died about twenty-four hours after fertilization, at the time when the embryo has begun to form and the germ-ring has covered about half of the egg. The *Ctenolabrus* eggs also died within twenty-four hours; but they had developed further in that time than the *Fundulus* hybrids, the natural rate of development being more rapid for *Ctenolabrus* than for *Fundulus*.

They reached a stage in which the embryo was fairly well formed and the germ-ring had gone two-thirds of the way around the egg.

The cytology of hybrids has attracted considerable attention in recent years. In 1904 W. J. Moenkhaus made a study of the hybrids between *Fundulus heteroclitus* and *Menidia notata*, which is of particular interest in the present connection. In the F. ♀ × M. ♂ cross, Moenkhaus finds that about 50 per cent of the eggs are dispermic, while in the reciprocal cross there are only a few which are poly- or dispermic. He did not succeed in keeping the hybrid larvae alive more than two or three days, and therefore made no study of their heredity. The most important part of the paper is the study of the germ-nuclei and the chromatin in the early cleavages. Moenkhaus says that the pronuclei fuse completely before the first cleavage, but he gives no figures to demonstrate that fusion. He passes at once from a figure which shows the pronuclei closely apposed— as he says, “about to fuse” to the metaphase of the first cleavage spindle. In this stage and in the anaphase, he finds two morphologically distinct kinds of chromosomes, each of which can be traced to one parent of the cross. His figures show the two types very plainly, and he traces these types as far as the late cleavage stage. At first the chromosomes are bilaterally grouped, that is, the paternal and maternal chromosomes are not mixed on the spindle, but in the third cleavage he finds this grouping destroyed, and as cleavage progresses the two types are completely mingled, though still persisting.

The work of D. H. Tennent ('08) on the eggs of *Toxopneustes* and *Arbacia* fertilized with *Moiria* sperm shows somewhat similar results. He finds that the pronuclei fuse completely; but in the Arb. ♀ × M. ♂ cross in which the two types of chromosomes are morphologically distinct, they are mingled, not grouped on the first cleavage spindle.

The paper of Jacques Loeb ('12) on heredity in heterogeneous hybrids forms a complement to the work of Moenkhaus, as it discusses the inheritance of maternal characters in hybrids between *Fundulus* and *Menidia*, *Ctenolabrus*, and *Stenotomus*

without taking up the cytology of such eggs. Loeb kept these hybrids alive a month or more, and they formed the heart, blood-vessels, eyes, and fins, though they never hatched. He believes that the sperm in these cases acts as a parthenogenetic agent, simply removing from the egg obstacles to its division. The hybrids are therefore 'pure breeds' and the abnormalities which occur are due to the interference with the normal chemical reactions caused by the introduction of a foreign sperm. These abnormalities are, he says, "in no sense hybrid characters" and he caused similar abnormalities by keeping normally fertilized *Fundulus* eggs in sealed Erlenmeyer flasks containing 50 cc. seawater + 2 cc. 0.01 per cent NaCN. In two instances, however, he finds in *Menidia* eggs fertilized with *Fundulus* sperm, red chromatophores such as are common in *Fundulus* eggs but are not present in normal *Menidia* eggs. This seems like genuine inheritance of a paternal character—the only evidence of such inheritance he has found in these experiments.

Many writers in discussing the purely maternal character of heterogeneous hybrids explain this one-sided inheritance by an elimination of the paternal chromatin which may take place in the early cleavages. Among these are Kupelwieser ('09), Baltzer ('10) and Tennent ('08 and '12). Kupelwieser fertilized sea-urchin eggs with mollusk sperm and found that although the eggs divided, the sperm had no part in this division. It lies at the side or at one pole of the spindle and probably degenerates in the 2-cell stage. He did not, at this time, get larvae old enough to show whether the characters were purely maternal or not. Later ('12) he fertilized *Echinus* eggs with the sperm of an annelid, and found that although the pronuclei almost always fuse before the first cleavage, the behavior of the male chromatin in the first cleavage spindle is always abnormal, and it is not included in the daughter-nuclei of the 2-cell stage. He traces the abnormalities of the larvae, which are maternal in character, to mechanical disturbances of the maternal chromatin caused by the undivided mass of male chromatin on the spindle.

Baltzer made various crosses between *Echinus*, *Strongylocentrotus*, *Sphaerechinus*, *Arbacia*, and *Antedon*, and found in many

cases that a definite number of chromosomes which he was often able to identify as paternal in origin were eliminated in the early cleavages. When this was the case, the larvae showed only maternal characters. When the male chromatin was retained the larvae were intermediate in character, except in the cases of *Echinus* ♀ × *Antedon* ♂ and Strong. ♀ × *Antedon* ♂ where there is no elimination of chromatin. In this case, nevertheless, purely maternal hybrids result. Tennent ('12) found much the same condition in hybrids between *Hipponoë* and *Toxopneustes*. In *Tox.* ♀ × *Hip.* ♂ there is no elimination of chromatin, and the male dominates; while in the *Hip.* ♀ × *Tox.* ♂ cross, in which the maternal characters dominate there is elimination of a certain amount of chromatin which, although it cannot be traced definitely to either parent, is presumably of paternal origin. In crosses between *Arbacia* and *Toxopneustes* ('12 b) he finds that some chromatin of female origin may also be eliminated.

In the hybrids with which the present paper is concerned, we find a condition differing somewhat from any of these, though naturally most like that described by Moenkhaus for the *Fundulus* × *Menidia* cross. As has been said, it is necessary to draw all conclusions from the *Fundulus* eggs as the material obtained from the reciprocal cross was unsuitable for study. The first stage of interest in the hybridized eggs of *Fundulus* is that represented in figures 1, 4 and 5, where the germ-nuclei are closely apposed, and the aster has divided. In figures 4 and 5 the asters lie at opposite poles of the pair of nuclei, while in figure 1 they have not yet taken that position. It will be noticed also that in one case the nuclei lie side by side, and in the other end to end. In many cases one can see a difference between the two nuclei in the density of the reticulum, but this is not universal, and it is impossible to say with any degree of certainty which is the male and which is the female. A study of the normal *Fundulus* egg shows that this failure of the germ-nuclei to unite is peculiar to the hybrids, and that the stage represented in figures 1, 4 and 5 is in fact that which immediately precedes the formation of the first cleavage spindle. The condition of the normally fertilized eggs is shown in figure 3. As long as the two

nuclei are separate the aster is undivided (see fig. 2) and it is only after complete fusion of the nuclei has taken place, as will be seen in figure 3, that the asters assume a polar position like that shown in figures 4 and 5.

The stage which immediately follows shows still more plainly that the pronuclei do not fuse in these hybrids. This stage is shown in figure 6, and the condition represented there has been found in all the hybrids of this stage. Figure 7 shows a normally fertilized *Fundulus* egg in a corresponding stage, and the difference between the two is obvious. In the hybrid there are two dark masses lying in the spindle, in which masses the chromosomes can be seen. In the normal egg a single mass of the same nature is present, evidently the fusion nucleus. Taken in connection with the total absence of single nucleus stages in the hybrid, the evidence shows that although the germ-nuclei fuse in the normally fertilized *Fundulus* egg, they do not do so when the egg is fertilized with *Ctenolabrus* sperm.

The chromosomes of *Fundulus* are much larger than those of *Ctenolabrus*. They vary somewhat in shape according to their stage of development, but they are always easily to be distinguished from the small round *Ctenolabrus* chromosomes. In the metaphase the *Fundulus* chromosomes are long, but not straight. They have usually a thickening at one end. This can be seen in figure 10 which shows a few chromosomes from a normal metaphase group. Figure 15 shows the *Fundulus* chromosomes in the anaphase, where they have lengthened and straightened. Figure 9 shows a spindle from a normally fertilized *Ctenolabrus* egg, with the typical round *Ctenolabrus* chromosomes.

In the hybridized *Fundulus* egg, these two types of chromosomes are easily seen in the first cleavage spindle. In the metaphase, indeed, the chromosomes lie so close together that the two types can hardly be distinguished, but as soon as they begin to draw apart one sees the difference very plainly. Of course in the study of these stages, sections should be selected in which all the chromosomes are included. Otherwise, small pieces of the *Fundulus* chromosomes might be confused with the *Ctenolabrus* chromosomes. Figure 11 shows a spindle in which the

chromosomes have begun to separate. In this case the types are grouped, the male chromosomes lying on one side and the female on the other side of the spindle; but this is not always the case. Figure 8 shows chromosomes from a similar stage in which the two types are mingled.

In the later anaphase the difference is still more evident. Here I find, as did Moenkhaus, that the foreign chromosomes sometimes lag behind the normal ones in going to the poles. Figures 12 and 17 show the anaphase of the first cleavage in two hybrid eggs, while figures 13 and 18 give the same stage for the normal eggs. The difference between the hybrid and normal eggs is distinct and constant except in the case shown in figure 19. This is drawn from an egg found on a slide that was supposed to have only normally fertilized eggs on it; but it is more reasonable to suppose that, by a mistake in the handling of the preserved material, a hybrid egg was included among the normal ones than that one normal egg should present an appearance so like the hybrids and so different from all the other normal eggs. Figures 14 and 16 show the chromosomes from the metaphase of the first cleavage of two hybrid eggs, drawn with a magnification of 2500 diameters. They should be compared with the corresponding stage in a normal egg which is represented in figure 15. Even in the smaller-scale drawings, the difference between the two chromosome groups is apparent, but in the figures drawn with the higher magnification the distinctions between two types are particularly apparent.

The telophase of the first cleavage in these hybrids is very much like the corresponding stage in the normal egg. The vesicles in some cases fuse a little more slowly as the foreign chromosomes have lagged a little in going to the poles; but the difference is slight, and by the time the asters have separated and come to a polar position preparatory to the second cleavage, the nuclei of hybrids and normals cannot be distinguished from each other. In both cases one can see traces of the vesicles in the nuclear reticulum: and this is the case in the resting nuclei of both hybrid and normal eggs throughout development. Figure 20 shows the telophase of the first cleavage of a normal egg, and

figure 21 the same stage for the hybrid; while figures 22 and 23 represent the nuclei of the completed 2-cell stage in hybrid and normal eggs respectively.

Figure 24 represents the prophase of the second cleavage in a hybrid *Fundulus* egg. It will be seen that there is here, as in a normal egg, a single mass of chromatic material on the spindle quite different from the double mass formed by the ununited germ-nuclei before the first cleavage. This is added proof that in the nuclei of the 2-cell stage, the paternal and maternal chromatin is mingled. That the types of chromosomes do not, however, lose their identity is shown by the fact that in the later stages of the second cleavage they appear as distinctly as they did in the first cleavage (fig. 25). Throughout the later cleavage, moreover, the resting nuclei and prophases of hybrid eggs are indistinguishable from those of the normal ones, while the hybrids continue to show the two types of chromosomes in the anaphase stages. Figures 27 and 28 and figures 29, 30, 31 and 32 illustrate this fact. Figure 27 is of a fourth cleavage spindle and figure 28 of a sixth, while the others are from 12-hour stages.

With such figures as these last ones before us we should hardly look for any considerable elimination of chromatin in the early stages, and in fact there is no evidence that the paternal chromatin is thrown out in these hybrids. Figure 26 shows the prophase of the fourth cleavage in a hybrid, and in this a small mass of chromatin is found outside the spindle, but there is no evidence that this is of paternal origin. This is the only case of such abnormality I have found in the early cleavages, and the evidence of the later stages leads one to think that the paternal chromatin is generally retained and divides normally.

The first appearance of abnormality that can be called general is in the 12-hour stage. Here we find often, in the middle of the blastoderm, large cells with irregular nuclei, in which there has evidently been a failure to divide normally. Figure 34 shows such a cell, and figure 33 two more, with a few normal cells drawn for comparison. But even here we cannot say that

there is certainly elimination of paternal chromatin; for in another part of the same egg in which these abnormal cells occur, spindles are to be found like those represented in figures 29 to 32, in which the *Ctenolabrus* chromosomes are present. The disturbance is undoubtedly a result of the fertilization with the foreign sperm—we find no such condition in normally fertilized eggs—but we cannot say that it concerns the paternal chromatin alone. It seems, rather, to involve the whole cell, and is probably the cause of the death of many of the embryos and the abnormalities of those that survive. Where the cells continue to divide normally they retain the two types of chromosomes.

It will be seen that the cytological study of these eggs hardly supports Loeb's theory that the function of the sperm in such hybrids is merely that of a parthenogenetic agent. Nor do the conditions agree with those found in Echinoderm hybrids, with the exception of those between the *Echinus* and *Strongylocentrotus* females and the *Antedon* male. Although up to the 24-hour stage the larvae show no signs of paternal inheritance, it would be hardly safe to say that they were purely maternal in character, if it were not for Loeb's description of later stages of hybrids between these same two species of fish. As he, however, found no paternal characters in these larvae at any time, it must be concluded that the *Fundulus* egg fertilized with the sperm of *Ctenolabrus* gives purely maternal larvae without elimination of the foreign chromatin.

SUMMARY

1. In the cross *Fundulus* ♀ × *Ctenolabrus* ♂ the germ-nuclei are closely applied to each other, but form chromosomes for the first division without having fused.
2. The chromosomes of the first cleavage spindle are of two types, which correspond respectively to those seen in the normally fertilized eggs of the two species.
3. Although the foreign chromosomes lag somewhat behind the others in going to the poles, they are all finally included in the daughter-nuclei of the 2-cell stage.

4. The two types of chromosomes reappear in the spindle of the second cleavage, and can be distinguished throughout the development, which was followed as far as the 12-hour stage.

5. In the 12-hour stage large cells with irregular nuclei are found, which represent the beginning of disturbances later shown by abnormality or death of the larvae.

6. There is no evidence of elimination of the paternal chromatin at any stage.

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After the present article went to press a paper by Gunther and Paula Hertwig on teleost hybrids appeared in the Archiv für Mikroskopische Anatomie (Bd. 84, Heft 2), the results of which agree in general with the above conclusions, though there is no case exactly parallel to that of *Fundulus* ♀ × *Ctenolabrus* ♂. The following points of agreement may be mentioned. The authors found no case of elimination of chromatin, and in most cases the paternal and maternal chromatin united, giving a perfectly normal cleavage. In all but one of the heterogeneous crosses, the larvae died in the blastula stage, or when the embryo was just beginning to form, and at this time the only cytological abnormality was the unusually large size of the nuclei. The case of *Crenilabrus* ♀ × *Box* ♂, however, is different from their other heterogeneous crosses, for in this case the larvae were very nearly normal. They were not, however, parthenogenetic larvae, as can be shown from the size of their nuclei, and from the fact that they inherit chromatophores such as are found in the male but not in the female parent. With the general conclusion that the disturbance involves the relation of nucleus and cytoplasm, rather than the nucleus alone, my results are in complete accord.

EXPLANATION OF PLATES

The essential features of all figures were drawn with a camera lucida. Unless otherwise stated, the magnification is 1000 diameters.

PLATE I

EXPLANATION OF FIGURES

- 1 F ♀ × C ♂; aster divided; germ-nuclei not yet closely apposed.
- 2 F ♀ × F ♂; germ-nuclei about to fuse; aster undivided.
- 3 F ♀ × F ♂; segmentation nucleus.
- 4 and 5 F ♀ × C ♂; germ-nuclei closely apposed but not fused
- 6 F ♀ × C ♂; prophase of first cleavage; the nuclei have not fused.
- 7 F ♀ × F ♂; prophase of first cleavage; the nuclei have fused
- 8 F ♀ × C ♂; early anaphase, first cleavage; paternal and maternal chromatin mingled (× 2500).
- 9 C ♀ × C ♂; metaphase, first cleavage.
- 10 F ♀ × F ♂; metaphase, first cleavage (× 2500).
- 11 F ♀ × C ♂, early anaphase, first cleavage; paternal chromosomes grouped on one side of the spindle.

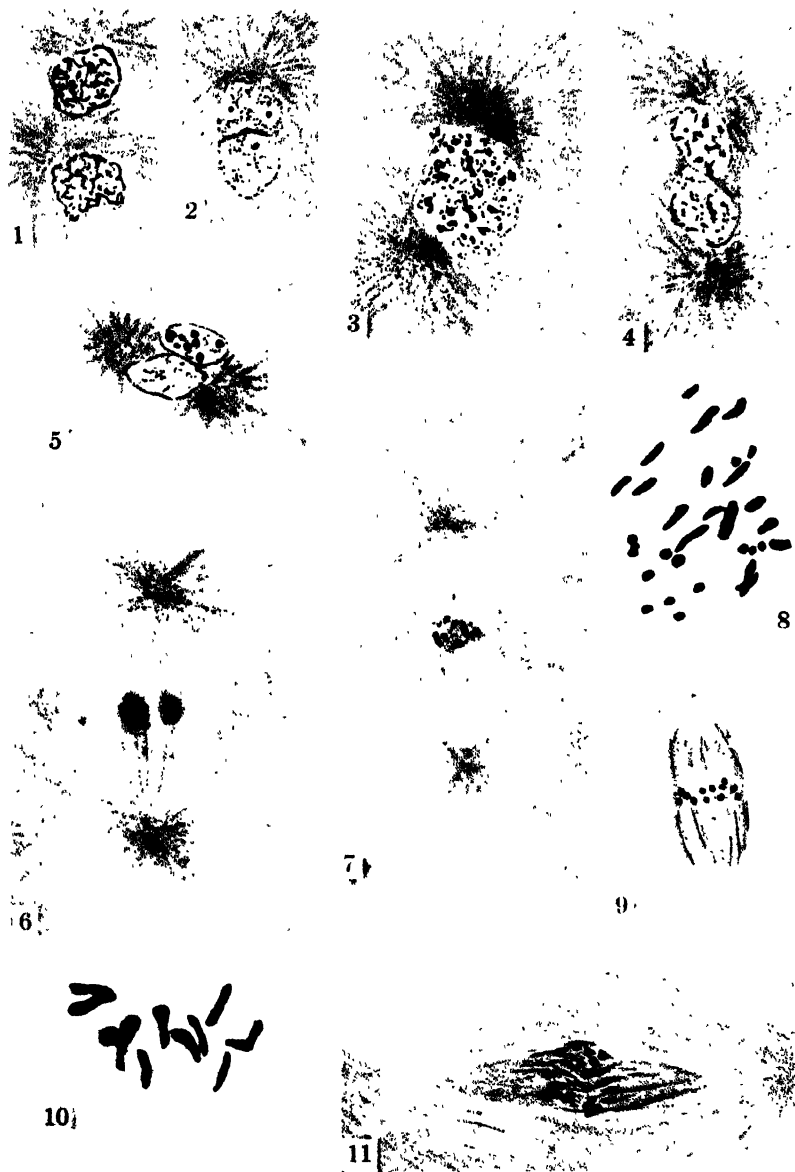


PLATE 2

EXPLANATION OF FIGURES

- 12 F ♀ × C ♂; anaphase, first cleavage.
 13 F ♀ × F ♂; anaphase, first cleavage.

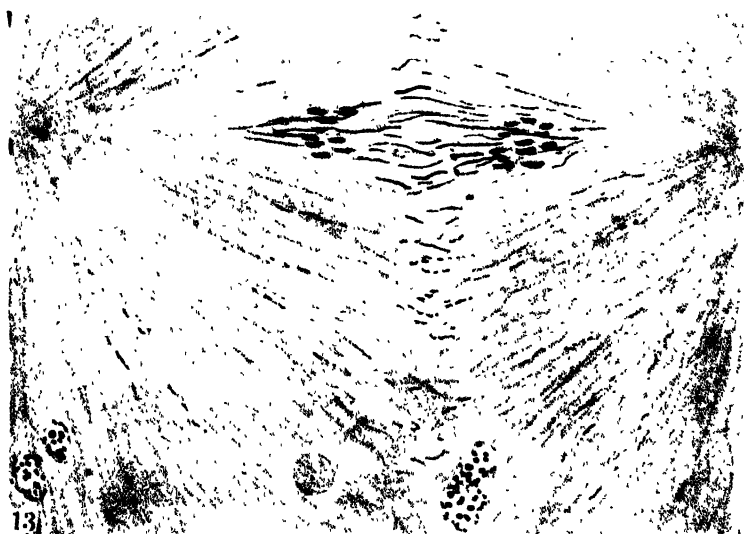
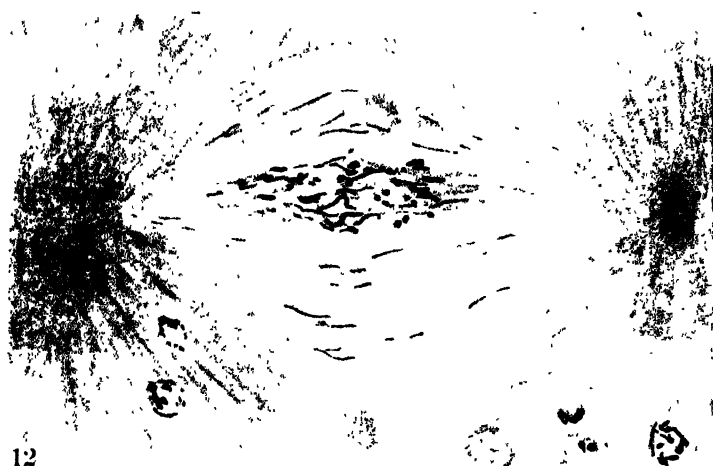


PLATE 3

EXPLANATION OF FIGURES

- 14 F ♀ × C ♂; anaphase, first cleavage (× 2500)
- 15 F ♀ × F ♂; anaphase, first cleavage (× 2500).
- 16 F ♀ × C ♂; anaphase, first cleavage (× 2500)
- 17 F ♀ × C ♂; anaphase, first cleavage.
- 18 F ♀ × F ♂; anaphase, first cleavage.
- 19 F ♀ × C ♂; anaphase, first cleavage.

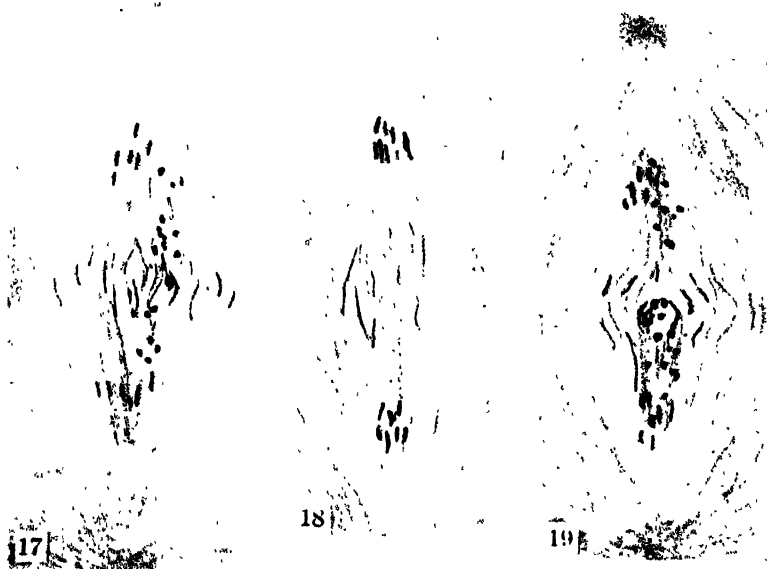
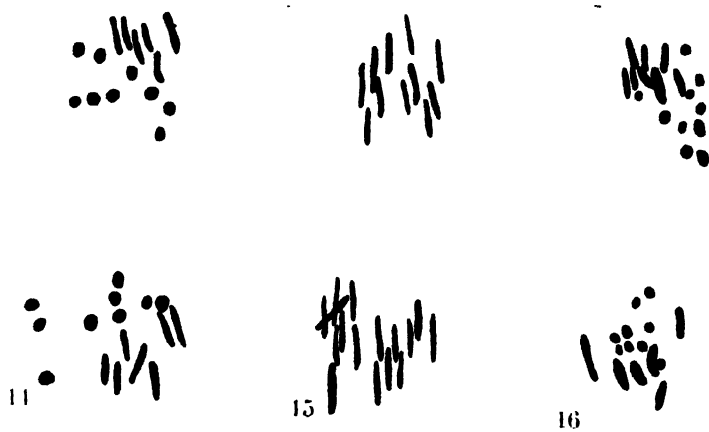


PLATE 4

EXPLANATION OF FIGURES

- 20 F ♀ × F ♂; telophase, first cleavage.
- 21 F ♀ × C ♂; telophase, first cleavage.
- 22 F ♀ × C ♂; nucleus, 2-cell stage.
- 23 F ♀ × F ♂; nucleus, 2-cell stage.
- 24 F ♀ × C ♂; prophase, second cleavage.
- 25 F ♀ × C ♂; anaphase, second cleavage.
- 26 F ♀ × C ♂; prophase, fourth cleavage.
- 27 F ♀ × C ♂; anaphase, fourth cleavage.
- 28 F ♀ × C ♂; anaphase, sixth cleavage.

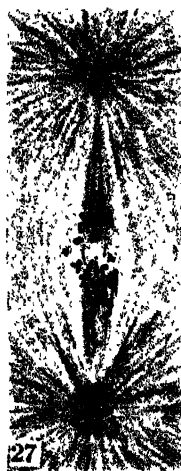
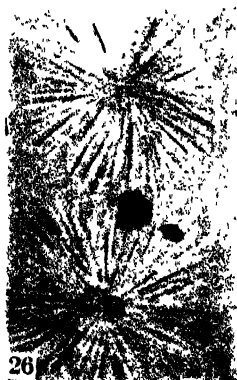
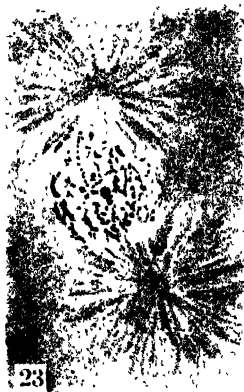
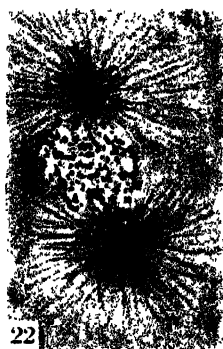
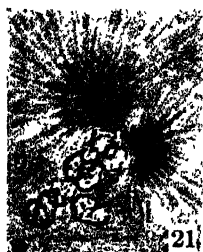


PLATE 5

EXPLANATION OF FIGURES

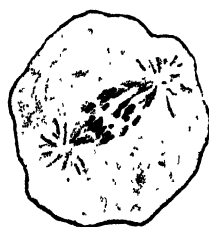
- 29, 30, 31 and 32 F ♀ × C ♂; normally dividing cells from 12-hour stages.
33 F ♀ × C ♂; large cells with irregular nuclei from 12-hour stage. A few normal cells are drawn for comparison (× 350).
34 F ♀ × C ♂; abnormal cell from 12-hour stage.
35 Fundulus sperm (× 2500).
36 Ctenolabrus sperm (× 2500).



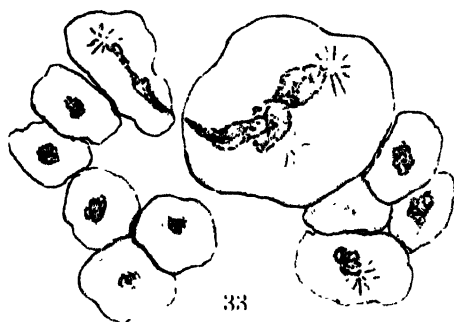
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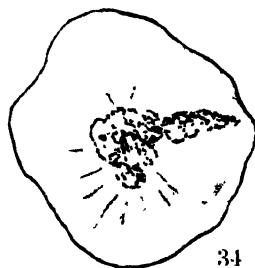
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STUDIES OF FERTILIZATION

VI. THE MECHANISM OF FERTILIZATION IN ARBACIA

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ONE FIGURE

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I. INTRODUCTION

The fifth study of this series on "The behavior of the spermatozoa of *Nereis* and *Arbacia* with special reference to egg extractives" forms the prelude to the present study. In that paper I dealt with the phenomena of activation and of aggregation (chemotaxis) of spermatozoa and with their agglutination by egg secretions of the same species. Regarding fertilization as involving chemical interaction of the ovum and the spermatozoon, I concluded from some of the modes of behavior of the spermatozoa, especially the agglutination phenomena, that "these small motile cells may prove better indicators of some of the reactions involved in fertilization than the slowly reacting egg" and that we might "confidently expect that study of the reactions of spermatozoa will break a new path into the field of fertilization."

The results described in the present paper have been obtained largely by the use of sperm-suspensions as indicators, but also by the study of an inhibitor of fertilization contained in the blood of certain individuals of the same species. These methods reveal a striking series of facts which appear to me to justify a somewhat new point of view in regard to some of the problems of fertilization. I have already briefly presented this point of view in a rather special form with an outline of the main facts in a preliminary paper (Lillie '13).

The essential conclusion is that fertilization is a reaction between three bodies of which one is born by the sperm and one by the egg; the third body, which is secreted by the egg, reacts with both the others. The spermatozoon functions essentially as an activator of the third body which I propose to name 'fertilizin';¹ the latter when activated enters into certain reactions in the cortex of the egg which lead to membrane formation. In order to give a concrete working conception I have pictured the fertilizin as possessing two side chains active in fertilization, viz: one

¹ This is the same substance which I called the 'sperm isoagglutinin' in the preceding paper of this series, because it causes agglutination in sperm suspensions of the same species.

reacting with the sperm which I call the 'spermophile side-chain' or group and the other reacting with the egg which I call the 'ovophile side-chain' or group. The chemical group of the sperm which reacts with the fertilizin is named the sperm receptor and that of the egg the egg receptor.

We are thus furnished with a concrete conception which answers very well for purposes of description; it need not however, be taken too literally. The theoretical aspect of the results is discussed in section IV.

The present paper presents in some detail the evidences on which this general conception of fertilization rests; it deals exclusively with *Arbacia*; in a later paper I shall present some confirmatory evidence from the study of *Nereis*. We shall consider first the action of the spermophile side-chain of the fertilizin and the evidence that the latter is a necessary link in the fertilization process. Second, we shall consider the nature of the ovophile side-chain of the fertilizin, the means of inhibiting its action and of neutralizing such inhibition.

The terminology has been largely adopted from immunology, because it seemed best suited to express the facts. If it seems rather bizarre to the zoölogical reader I must ask him not to conceive prejudice for this reason against the facts themselves, which stand on their own feet quite apart from any terminology or theory. The terminology and the theory are significant only to the extent that they give a brief description of the facts, and serve as a working hypothesis.

II. THE SPERMOPHILE SIDE-CHAIN

I. A QUANTITATIVE METHOD OF STUDYING THE PRODUCTION OF FERTILIZIN BY OVA

The fundamental fact which forms our point of departure is that fertilizable eggs of *Arbacia* suspended in sea-water secrete a substance (fertilizin) for which only the spermatozoa of the same species constitute an efficient indicator. Similarly the only cells that produce the substance are the egg-cells of the same species; it is not found in the blood or in extracts of other tissues.

The presence of the substance is shown by the agglutination of sperm-suspensions of the same species. If a few drops of sea-water containing this substance in sufficient concentration be added to a 1 per cent sperm-suspension in a test-tube a flocculent condition becomes apparent to the naked eye in a few moments. The qualitative aspects of the reaction were described quite fully in the preceding paper of this series, and need not therefore be repeated here.

In my previous papers ('12 and '13) no attempt was made to establish a quantitative method of study. But in order to carry the subject farther it was necessary to determine how much of the sperm agglutinating substance was produced by the ova under various conditions. As noted in previous papers the reaction is reversible. With high concentrations of the agglutinating substance it may be several minutes before the agglutinated masses break up and reversal is complete; with low concentrations on the other hand the agglutinated masses are smaller and their disintegration is correspondingly more rapid. It was therefore possible to establish as a unit concentration of the agglutinating substance the greatest dilution at which an unmistakable reaction is given. Such a reaction lasts only a few seconds. It would be obviously very tedious to establish the *exact* point at which the reaction ceases in a long series of experiments, and in practice I prepared a series of dilutions each member of which was one-half of the preceding member, and the last one to give the reaction was regarded as the unit concentration. The method is as follows: Taking a given solution of the fertilizin a series of dilutions is made. If these begin with (a) 1/100 (i.e., 99 parts of sea-water plus 1 part of the solution) a half dilution of (a) is made, (b) (i.e., 1/200); a half dilution of (b) follows (c) 1/400, (d) 1/800, (e) 1/1600, (f) 1/3200, etc. If 1/1600 is negative, 1/800 may give a 4 to 6-second reaction; if 1/800 is negative 1/400 may give a 4 to 6-second reaction, etc; when the solution was known to be strong it was usual to begin with a 1/100 dilution; otherwise we might begin with a 1/10 dilution, followed by a 1/20, 1/40, etc., or 1/2 followed by 1/4, 1/8, 1/16, etc., the last member of a series to give a positive reaction was accepted as

of unit concentration. Thus any given solution could be rated as of 10, 100, or 6400, etc., agglutinating strength depending on the dilution test.

The most delicate method of determining the reaction is the raised cover-slip method described in a previous paper ('13). The sperm-suspension used as indicator should be about 1 per cent (i.e., one drop dry sperm to about 99 drops sea-water) and should be not more than 10 minutes old. A few drops of this are mounted on a glass slide beneath a long cover-slip supported by glass rods about 1 to 1.5 mm. in diameter. The slide is placed on the stage of a microscope in focus under a magnification of about 40 diameters and a drop of the solution to be tested is blown into the sperm suspension with a capillary pipette attached to a flexible rubber tube. The agglutination reaction begins within less than a second if the solution be of considerable agglutinating power, or within 2 or 3 seconds if the solution is diluted nearly to the unit reaction. In the latter case it lasts only 4 to 6 seconds. The masses formed are relatively small in the latter case, and larger and denser proportionately with the higher concentrations.

The reaction is accompanied by a strong stimulation of the spermatozoa, and the reversal of the reaction by slackening of movement, which is more or less proportional to the strength of the solution, so that after agglutination with a strong solution the spermatozoa are practically paralyzed for a time. For this reason the agglutination masses formed by a strong solution do not break up readily and it is difficult to fix the exact cessation of the reaction; but with concentrations up to about 20 units the beginning and end of the reaction are both clearly marked. For a more detailed account of the reaction with higher concentrations, see Study V (Lillie '13, p. 550).

As an important detail of technique, I would say that for the determination of the more delicate reactions it is desirable to have the sperm suspensions free from blood corpuscles or other foreign particles. I therefore proceed as follows: the sea-urchin is dried slightly with a towel and is opened by a circular cut on the oral side of the equator. If it be a ripe male the thick sperm will begin to exude from the genital pores. The animal is then

laid aboral side down in a Syracuse watch crystal and the sperm allowed to collect in the crystal. In this way, as much as 2 cc. of thick perfectly clean sperm may be obtained from a large ripe male. If a few drops of sea-water have dripped off with the sperm it may be removed with a pipette, but if the sperm is abundant it is so dense that the fluid lies on it without intermingling. Such a mass of sperm will keep perfectly fresh for several hours, and suspensions may be made from it as needed for the tests. But the suspension used in testing must be freshly prepared if delicate reactions are desired.

The fertilizin is actively secreted by the eggs in sea-water as noted beyond; the concentration in the fluid of a given suspension of eggs in sea-water is a function of the quantity of eggs in the suspension. It will be convenient, therefore, in referring to any given agglutination solution, to have a method of expressing the relative quantity of eggs in proportion to the sea-water. The most convenient way of expressing this relation is to express the bulk of eggs as a percentage of the entire suspension. Thus 1 cc. of eggs added to 9 cc. of sea-water will be denominated a 10 per cent egg suspension, etc. This form of expression was not adopted until after the experiments were done; the figures are, therefore, not always even numbers, and are not intended in any case to be absolutely exact, but this is a matter of little importance as the density of a given egg suspension is only one factor in the concentration of the fertilizin in it; the condition of the eggs, whether entirely ripe or immature, fresh or stale, with or without jelly, is more important.

2. THE QUANTITY OF FERTILIZIN PRODUCED BY OVA OF ARBACIA

a. By a single washing

July 6, 1913: For this experiment I used the eggs of a single female shed in a watch crystal in the same manner as described above for the collection of clean sperm. The eggs were thus free from blood or fragments of the ovaries, and contained only a trace of the sea-water. Graded quantities of eggs were put in a series of six test-tubes containing 5 cc. of sea-water each. The eggs were placed in the tubes at 9:45 A.M.; each tube was turned upside down six times and the eggs then allowed to

settle; there was no shaking or rough handling of the eggs, so that they were absolutely uninjured. At 10:20 A.M., after the eggs had settled to the bottoms of the tubes, samples of the sea-water in the tubes 1 to 5 were removed and tested by a series of dilutions; at 11:45 A.M. a sample of 6 was taken and tested. The concentration of the sperm agglutinating substance in each is shown in table 1.

TABLE 1

TUBE	EGG SUSPENSIONS PER CENT	AGGLUTINATING STRENGTH OF SUPER- NATANT SEA-WATER	TESTS	
			Dilutions	Reactions
1	0.5	50	1/50	4-5 seconds
2	1.0	100	1/100	4-5 seconds
3	2.0	100+	1/100	10 seconds
4	4.0	400	1/400	6 seconds
5	8.0	800	1/800	6 seconds
6	16.6	1600	1/1600	7-8 seconds

It is interesting to note that the quantity of fertilizin produced is so nearly proportional to the quantity of eggs. (No. 3 would probably have tested to 1/200 with perfectly fresh sperm.) We might expect in general that the quantity of fertilizin produced would continue to be proportional to the quantity of eggs. This however, may not be the case, as there are indications that in the presence of a certain concentration in the sea-water more is not secreted by the eggs.

It would appear that after the eggs had settled in the tubes into a dense layer on the bottom they ceased to produce more agglutinating substance, because the concentration in tube 6 in which the sea-water had stood over the eggs much longer than in tubes 1 to 5 was proportional to the quantity of eggs and not to the time.

Higher concentrations of the fertilizin were often obtained: August 9: A 25 per cent egg suspension stood for 12 minutes. The supernatant fluid then gave the following sperm-agglutination tests: diluted to 1/6400, 7-second reaction; 1/3200, 15-second reaction. August 18: egg suspension (density not given) was shaken. After settling of the eggs the supernatant fluid

gave the following sperm-agglutination tests: diluted to 1/12800, 5-second reaction; 1/6400, 10 to 12-second reaction.

Enough has been said to indicate the large amounts of this substance produced by the eggs in sea-water. No attempt has been made to produce the highest attainable concentration. The standardization of solutions for experiments was a daily procedure and in the experiments themselves hundreds of tests were made.

Hitherto, I think, the unfertilized egg has been generally regarded as a very inert cell, almost in a state of suspended animation. But it is necessary to modify such a point of view very greatly. The egg of *Arbacia*, at least, is a very actively secreting cell. Although the method which I have used enables us to calculate only the relative concentration of the agglutinating substance produced, yet the quantity must be very considerable when we consider that in tube 1, of the preceding experiment the egg suspension was only 0.5 per cent and that the supernatant fluid was charged with 50 units of the agglutinating substance in 35 minutes.

b. Comparison of ripe and unripe eggs

The tests already given are from fresh, ripe eggs. Ovaries containing a high proportion of ovocytes produce less fertilizin proportionately; but such ovaries could be obtained only at the beginning of the season so that only a few tests were made. On June 16 I compared equal amounts of ripe ovaries with practically all eggs mature, and unripe ovaries with only 50 per cent of the eggs mature. One part finely cut up ovary was added in each case to 3 parts sea-water. The first tested to 1/612, the second to 1/64. In a second determination the same day, in which *a* had only 5 per cent unripe eggs (ovocytes with germinal vesicle) and *b* had 50 per cent, one part ovary to 2 parts sea-water in each case, the tests were *a* 1/1000, *b* 1/600. In the latter case the strength of the solutions was about proportional to the percentage of mature eggs. In the former case the unripe ovary did not give as large a production of the fertilizin as would be expected

from the relative proportions of ripe eggs. Presumably, the ovarian tissue was relatively more bulky in this case.

The ovocytes are surrounded with the same layer of jelly in *Arbacia* as the mature eggs; hence we must conclude that the jelly is not the source of the agglutinating substance to whatever extent it may become charged with it after maturation. An important point is involved in this conclusion discussed farther on.

I think we are justified in concluding that ovocytes do not secrete the agglutinating substance. The conditions in *Nereis* fortify this conclusion, inasmuch as the ovocytes with intact germinal vesicle do not secrete the fertilizin, but as soon as insemination takes place it is poured out abundantly and the germinal vesicle breaks down.

In this connection it is important to note that in some animals ovocytes with intact germinal vesicle do not respond to the spermatozoon, even if cut in two so that the protoplasm is laid bare. Wilson ('03) has shown this for the nemertean, *Cerebratulus*, and Delage ('01) for the eggs of *Strongylocentrotus* and *Asterias*. As I show farther on that the fertilizin is necessary for fertilization, we may find in these facts evidence that the fertilizin is not only not secreted but is not even preformed in ovocytes with intact germinal vesicle, at any rate not in the forms mentioned. Its absence would furnish sufficient explanation of the failure of such egg-fragments to fertilize, and the consequent impossibility of producing merogony until the germinal vesicle breaks down.

Delage ('01) also shows that, as soon as the germinal vesicle begins to fade, in *Asterias*, and the nuclear sap diffuses into the cytoplasm, the egg becomes fertilizable and merogony can be induced. According to my interpretation the formation of fertilizin must begin at this time. At any rate the non-fertilizable condition of the cytoplasm before the germinal vesicle begins to fade is associated with the absence of fertilizin.

It is not necessary to assume that the fertilizin is contained in the nuclear sap; on the contrary, the long-continued production of this substance, as described in the next section, runs counter to this idea. It is more probable that it exists in a state comparable to zymogen ready to be converted into active form, and

that the escape of nuclear sap furnishes the necessary conditions. In some eggs, therefore, we might anticipate that sufficient substance might exude from the germinal vesicle without rupture to bring about formation of active fertilizin. Theoretically this should occur in such eggs as those of *Nereis* in which fertilization occurs before rupture of the germinal vesicle.

c. Fertilizin produced in a series of washings; rôle of the jelly

The production of this substance by the egg, in sea-water must be regarded as an active process of secretion, and, as will be seen immediately, it goes on continuously, in the case of *Arbacia*, as long as the eggs remain alive.

The last fact was ascertained in several attempts to remove all the fertilizin from the eggs by repeated washings. In these experiments it was also ascertained that while the jelly layer surrounding the eggs is saturated with fertilizin, eggs deprived of jelly still continue to produce it in considerable quantities. The method of making such tests is to suspend a measured amount of eggs in a given amount of sea-water and allow them to settle; as much of the supernatant fluid is then poured off as is possible without losing any eggs and kept for testing, and an equivalent amount of fresh sea-water replaced; the eggs are mixed up thoroughly, and allowed to settle, and the process is continued. In one series running three days in which the quantity of eggs was originally 2 cc. and the total volume of sea-water and eggs in the tube 10 cc., 6 to 8 cc. being poured off at each settling, thirty-four changes were made, and the agglutinating strength of the supernatant fluid diminished from 100 at first to 20 at the end. The details are given in table 2: Under the heading 'washings' the denominator gives the quantity of eggs and sea-water left after pouring off the supernatant fluid, and the numerator the total quantity after addition of the fresh sea-water.

This long series is amazing in its revelation of the extraordinary energy of the eggs in producing the fertilizin. In this case the eggs were allowed to retain their jelly, which, however, slowly dissolved until it was nearly all gone in washing 24.

TABLE 2

WASHINGS	TESTS	BULK OF EGGS	TIME OF CHANGE
1913			
August 14			
1 10/3.0 cc.	1/100—10-12 sec.	2.0 cc.	11:01 A.M.
2 10/2.5 cc.	1/100 — 8 sec.		
3 10/2.5 cc.	1/50 —10-11 sec.	1.15 cc.	11:12 A.M.
4 10/2.0 cc.	1/50 — 10 sec.	1.1 cc.	11:22 A.M.
5 10/2.0 cc.	1/100 — 6 sec.		11:32 A.M.
6 10/2.0 cc.	1/50 — 9 sec.	1.2 cc.	1:55 P.M.
7 10/1.7 cc.	1/100 — 12 sec.	0.9 cc.	2:20 P.M.
8 10/1.7 cc.	1/100 — 7 sec.		2:35 P.M.
9 10/1.7 cc.	1/100 — 7 sec.		2:45 P.M.
10 10/1.7 cc.	1/100—11-13 sec.	0.8 cc.	2:53 P.M.
11 10/2.0 cc.	1/100 — 11 sec.	0.8 cc.	3:03 P.M.
12 10/1.7 cc.	1/100 — 8 sec.	0.8 cc.	3:13 P.M.
13 10/1.5 cc. ¹	1/100 — 12 sec.		3:23 P.M.
14 Fluid not changed ²	1/100 — 11 sec.		4:12 P.M.
August 15			
14a 10/2.8 cc.	1/50 slow atypical reaction		8:18 A.M.
15 10/1.9 cc.	1/100 — 12 sec.	0.7 cc.	9:20 A.M.
16 10/2.0 cc.	1/100 — 13 sec.	0.7 cc.	9:56 A.M.
17 10/2.0 cc.	1/100 — 7 sec.	0.65 cc.	10:22 A.M.
18 10/2.0 cc.	1/25 — 7 sec.		10:40 A.M.
(Note sudden fall 17-18)			
19 10/2.0 cc.	1/50 — 5 sec.		11:04 A.M.
20 10/2.0 cc.	1/25 — 5 sec.	0.6 cc.	11:34 A.M.
21 10/2.0 cc.	1/20 — 9 sec.	0.5 cc.	2:15 P.M.
22 10/4.0 cc.	1/20 — 7 sec.		2:45 P.M.
23 10/ .0 cc.	1/20 — 7 sec.	0.55 cc.	3:45 P.M.
24 10/2.0 cc.	1/20 — 7 sec.		4:25 P.M.
No jelly left on the eggs, except a thin layer on a few			
August 16 Continued the above series to 33 washings from			9:08 A.M. to
4:05 P.M.			
25			
26			
27	1/20 — 7 sec.		
28			
29			
30 ³			
31	1/20 — 5 sec.		
32	1/10 — 10 sec.		
33	1/20 — 18 sec.		

¹ The jelly is now dissolved until only a thin layer is left. Washings 3-14 were absolutely colorless; and the following ones remained so until 35.

² The eggs were precipitated by slight centrifuging in the first 13 washings, except 6; in the others they were allowed to settle. The gradual reduction in bulk of the eggs is due to loss of jelly, not to loss of eggs.

³ A drop from No. 30 was fertilised with excess of sperm at 11:36 A.M.; 5 per cent only segmented, irregularly; no membranes formed.

TABLE 2—Continued

WASHINGS	TESTS	BULK OF EGGS	TIME OF CHANGE
1913			
August 17			
34 12/1.5 cc.	1/20 — 5 sec.	0 325 cc.	9:15 A.M.
35	On stirring up, the eggs disintegrate, and the fluid becomes highly colored; the agglutinating substance is <i>absolutely neutralized</i> . Test 1/1 negative, but the fluid is highly chemotactic and activating.		

In order to determine the rôle of the jelly in charging the seawater with fertilizin, I killed some eggs by heating them for 5 minutes to 60° C. (August 15, 1913,) and submitted them to a series of 22 washings in 3 days, by which time the jelly was nearly all dissolved away: the agglutinating power decreased from 800 at first to 1 at the end. The agglutinating substance could be detected as long as any jelly remained.

On the other hand, eggs may be entirely deprived of jelly by shaking, and such eggs will continue to produce fertilizin as long as eggs with jelly, though in lesser quantities at first. This matter is so fundamental that I include another long table giving a comparison of eggs deprived of jelly by shaking and those with jelly (table 3).

August 21, 1913: In this experiment ripe eggs of *Arbacia* were washed once and divided in three equal lots, *A*, *B*, and *C*, in 10 cc. seawater in graduated cylinders. They were allowed to settle, and at 9:20 A.M. the bulk of eggs was 1.8 cc. in *A*, 2 cc. in *B*, and 2 cc. in *C*. Thus there were about 10 per cent fewer eggs in *A* than in *B* and *C*. At 9:23 *A* was transferred to a test-tube and given 6 vigorous shakes to get rid of the jelly. A sample was then examined in India ink and it was found that 90 per cent of the eggs were entirely freed from the jelly; in the remainder it was usually reduced to a thin layer. *A* was then returned to its cylinder in 10 cc. seawater and allowed to settle. As the eggs settled in *A* it could be seen that the fluid was only slightly colored; so that it was certain that not many eggs were injured in the shaking.² At 9:40 A.M. the bulk of eggs was as follows: *A*, 0.6 cc.; *B*, 1.6 cc.; *C*, 1.6 cc. As care was taken to avoid loss of eggs in *A* the

² It should be noted that in such an experiment, it is extremely important not to shake too much, for if the eggs themselves are injured so that the color escapes, a complicating factor comes in considered beyond (see section 8, p. 544).

decrease in bulk is due to loss of jelly, in *B* and *C* simply to further settling. *C* was kept for control. *A* and *B* were submitted to repeated washings as in table 3, p. 536.

This experiment positively decides the question as to production of fertilizin. It continues to be produced by the unfertilized eggs without jelly as long as they live. The jelly is, naturally, saturated with fertilizin, and it seems probable that it may act to prevent too rapid exhaustion of the secreting mechanism of the egg. It is of course possible that all that the egg gives off is preformed, but it is more probable that loss of this substance by the egg acts as stimulus to regeneration of more.

Apparently the eggs do not secrete the fertilizin in such a series of washings after they have settled in a mass at the bottom of the tube; for it will be noticed that the concentration of the substance in the successive washings is not a factor of the time intervals. On the contrary, a long time interval between washings is often associated with a temporary decrease in concentration, as may be noted, for instance, in the interval of 15 hours between 14 and 14a in table 2. In this case 14 and 14a are actually the same body of supernatant fluid and the concentration is much decreased in the second test 15 hours after the first, as though the eggs were gradually destroying the fertilizin. This subject is fully treated in section 6 (anti-fertilizin).

3. THE BINDING OF FERTILIZIN BY SPERM

In my previous paper (Lillie '13) I showed that the spermatozoa fix the agglutinating substance, and assumed as a working hypothesis that the fixation was due to chemical union. The fact on which the statement was based was the disappearance of the agglutinating substance from a sperm suspension when not present in excess. No attempt was made, however, to make quantitative determinations, so the matter was taken up again in 1913 in a more exact manner. The amount of the agglutinating substance actually fixed by the sperm is surprisingly small when the great avidity of the sperm for it is taken into account.

TABLE 3

WASHINGS		TESTS	BULK OF EGGS	TIME OF CHANGE
1913				
August 21				
1	A 10/2.0		0.6 cc.	9:42 A.M.
	B 10/3.0		1.6 cc.	9:42 A.M.
2	A 10/2.0		0.6 cc.	10:18 A.M.
	B 10/2.4		1.4 cc.	10:18 A.M.
3	A 10/2.6		0.55 cc.	10:40 A.M.
	B 10/3.0		1.4 cc.	10:40 A.M.
4	A 10/2.4		0.55 cc.	11:04 A.M.
	B 10/2.7		1.3 cc.	11:04 A.M.
5	A 10/3.0		0.55 cc.	11:27 A.M.
	B 10/2.6		1.2 cc.	11:27 A.M.
6	A 10/2.0		0.55 cc.	11:47 A.M.
	B 10/2.7		1.2 cc.	11:47 A.M.
7	A 10/2.0		0.5 cc. plus	2:03 P.M.
	B 10/1.6		0.8 cc.	2:03 P.M.
8	A 10/2.5	1/10 neg.; 1/1—50 sec.		2:30 P.M.
	B 10/2.5	1/20—13 sec.		
9	A 10/2.0			3:15 P.M.
	B 10/2.0			3:15 P.M.
10	A 10/2.0			3:45 P.M.
	B 10/2.3			3:45 P.M.
11	A 10/2.0			4:06 P.M.
	B 10/2.4			4:06 P.M.
Fertilization 1				
12	A 10/2.0			4:40 P.M.
	B 10/2.5			4:40 P.M.
13	A 10/1.5			7:32 P.M.
	B 10/1.2			7:32 P.M.
August 22				
14	A 10.4/3.4		0.4 cc.	8:18 A.M.
	B 10/2.8		0.6 cc.	* 8:18 A.M.
15	A 10/1.4			8:38 A.M.
	B 10/2.0			8:38 A.M.
16	A 11/1.8	1/10 trace; 1/1—60 sec.		9:13 A.M.
	B 11/2.2	1/10—20 sec.		9:13 A.M.
Fertilization 2				
17	A 10/1.7			9:40 A.M.
	B 10/2.5			9:40 A.M.
18	A 10/1.0			10:05 A.M.
	B 10.5/2.4			10:05 A.M.
19	A 10/1.0			10:23 A.M.
	B 10/3.0			10:23 A.M.

TABLE 3—Continued

WASHINGS		TESTS	BULK OF EGGS	TIME OF CHANGE
1913				
August 22				
20	A 10/1.0	1/10 trace; 1/1 trace and strong chemotaxis		10:48 A.M.
	B 10/2.4	1/10—9 sec.		
Note: Some eggs going to pieces in A				
21	A 10/1.5			11:10 A.M.
	B 10/3.0			
22	A 10/1.0			11:30 A.M.
	B 10/2.2			
23	A 10/1.4			2:12 P.M.
	B 10/2.0			
24	A 10/1.4	1/1 trace and strong chemotaxis		2:33 A.M.
	B 10/1.8	1/1—25 sec., slight chemotaxis		
25	A 10/1.0			2:55 P.M.
	B 10/1.2			
26	A 11/1.0			4:03 P.M.
	B 11/2.0			
27	A 10/1.0			4:19 P.M.
	B 10/2.0			
Fertilization 3				
28	A 10/2.0			4:36 P.M.
	B 10/1.0			
29	A 10/1.0			7:45 P.M.
	B 11/2.0			
August 23				
30	A 10/1.0	1/1—20 sec., chemotaxis ¹		8:37 A.M.
	B 10/2.5	1/1—30 sec., no chemotaxis		
31	A 10/1.5	1/1—14 sec. strong chemotaxis		9:13 A.M.
	B 10/2.0	1/1—20 sec. strong chemotaxis		
Fertilization 4				
32	A 10/1.0			10:10 A.M.
	B 10/2.0			
33	A 10/1.0	1/1—11 sec. strong chemotaxis		11:30 A.M.
	B 10/1.6	1/1—11 sec. strong chemotaxis		

¹ The chemotaxis noted in this series is due to injured eggs which secrete anti-fertilisin and conceal the fertilisin. See discussion.

TABLE 3—(continued)

WARNINGS	TESTS	BULK OF EGGS	TIME OF CHANGE
1913			
August 23			
34 A 10/0.5			2:36 P.M.
B 10/1.0			
35 A; fluid not changed	1/1—11 sec.		
B fluid not changed	1/1—10 sec.		
August 24			
36 A; fluid not changed	1/1—8 second reaction		9:35 A.M.
B fluid not changed	1/1—7 second reaction		
37 Eggs shaken	and broken; fluid not changed.		9:42 A.M.
A	1/1—negative		
B	1/1—4 sec.; 2d test negative		

Thus on August 13, I standardized an agglutination solution in sea-water to about 128 agglutinating power and placed 1 cc. in a tube (A). To this was added 1 cc. of a 3.3 per cent sperm suspension; 1 cc. of the agglutinated mixture *A* was then added to tube (B) containing 1 cc. of the same sperm suspension; 1 cc. of *B* was then added to 1 cc. of the same sperm suspension in tube (C). Thus the 128 power agglutinating solution was diluted with 3.3 per cent sperm twice in *A*, four times in *B*, and eight times in *C*.

(A) 1 cc. 128 agglutinating power solution plus 1 cc. 3.3 per cent sperm

(B) 1 cc. A plus 1 cc. 3.3 per cent sperm

(C) 1 cc. B plus 1 cc. 3.3 per cent sperm

The sperm was then centrifuged off in the three tubes and on test the supernatant fluid was negative in *C*, faintly positive in *B* and gave a 60 to 80-second reaction in *A*. On renewed test, *B* was found almost negative. The result is then that 1 cc. of a 128 power agglutinating solution may be fixed by 3 cc. of 3.3 per cent sperm.

Other tests of the binding power of sperm gave results of the same order of magnitude. Thus on August 9, I used a 64 power agglutinating solution as follows:

- (A) 1 cc. 64 agglutinating solution plus 1 cc. (approx.) 3 per cent sperm
- (B) 1 cc. A plus 1 cc. 3 per cent sperm
- (C) 1 cc. B plus 1 cc. 3 per cent sperm
- (D) 1 cc. C plus 1 cc. 3 per cent sperm

In this case after centrifuging off the sperm, *B*, *C* and *D* were negative; *A* was faintly positive. That is, 1 cc. of approximately 3 per cent sperm fixed 1 cc. of a 64 power agglutinating solution. This shows a greater binding power than in the previous experiment, but easily within the limits of error arising from difficulty of standardizing the sperm suspension, owing to the unavoidable inclusion of some fluid in the sperm. Other tests gave comparable results. Reduced to common terms:

August 9: 1 cc. 3 per cent sperm fixes 1 cc. 64 power agglutinating solution.

August 13: 1 cc. 3.3 per cent sperm fixes 0.66 cc. 64 power agglutinating solution

The difference is considerable, but when one considers first the difficulty of obtaining equivalent sperm suspensions and second the 2 to 1 method of standardizing the agglutinating solutions, and third, possible variations in the delicacy of the sperm suspensions used as indicators, the results lie well within the limit of error and indicate that there is a definite quantitative relation involved.

The possibility remains that the disappearance of the agglutinating substance in sperm suspensions is a phenomenon of adsorption rather than of chemical union. Experiments planned for an adequate test of this possibility were crowded out by other problems.

If sperm suspensions are allowed to stand for some hours they reach a condition when they will agglutinate only with strong solutions. If the binding power is then tested it is found that they fix as much of the agglutinating substance as before. The fixing power of the sperm is thus entirely independent of its capacity for being agglutinated, which is of course to be expected. But it is important, nevertheless, to emphasize it; the capacity for being agglutinated depends upon the motility of the spermatozoa, the binding capacity depends merely upon the presence of

chemical side-chains (receptors) capable of uniting with the agglutinating substance. When such a union takes place the heads of the spermatozoa must be supposed to become adhesive; if they move about rapidly, there are more collisions and hence more agglutination. With stale spermatozoa, either the movements are too slow to produce much agglutination, or the receptors are cast off and lie free in the medium, so that their binding does not affect the physical properties of the spermatozoa themselves. The latter possibility should be investigated, but was not this summer owing to the multitude of other problems.

A little light may be thrown on this problem by the results of June 26: In that case 4 drops of a strong, but not definitely standardized, solution of the agglutinating substance was added to 4 cc. of a 3 per cent sperm suspension 3 hours old. The tube was then centrifuged strongly and most of the spermatozoa precipitated in a mass at the bottom; however the supernatant fluid remained opalescent. Tested immediately with a fresh sperm suspension the fluid gave a 17 to 25-second agglutinating reaction. The tube was allowed to stand five minutes longer and was then tested again; the precipitated sperm remained in a solid mass, but the supernatant fluid had become negative; it contained no more free agglutinating substance. The quantity of sperm in suspension seemed much too small to account for the neutralization, which I am inclined to attribute to free receptors. But no really adequate test was made.

As to the interpretation to be given to the reversal of agglutination of the sperm it may be supposed either, (1) that the adhesive condition on which the agglutination depends exists only during an early union of the agglutinating substance with the sperm receptors and is lost as the union becomes firmer; or (2) that the bound receptors are cast off by the sperm. In view of the nature of the mechanism of the fertilization process, discussed beyond, I am inclined to lean to the second hypothesis.

4. SEPARATENESS OF HETERO-AGGLUTINATING SUBSTANCES IN EGG-SECRETIONS

I stated in my previous paper that in strong agglutinating solutions from *Arbacia* eggs and ovaries there is present also a substance that agglutinates the sperm of *Nereis*, and I argued that this was probably distinct from the iso-agglutinating substance because (1) it is contained in the blood of *Arbacia* which is normally entirely free from the iso-agglutinating substance, and (2) it may disappear from agglutinating solutions on standing while the iso-agglutinating substance remains apparently intact.

Some farther experiments made this year demonstrate the fact that the substance in agglutinating solutions from *Arbacia* eggs, that attacks *Nereis* sperm is a separate substance. We may note in the first place that the effect of this substance on the *Nereis* sperm is distinctly toxic. A drop containing it injected into a *Nereis* sperm suspension beneath a raised cover slip causes the formation of a permanent coagulated ring at the margin of the drop. In this respect the action differs from the iso-agglutination which is without toxic effects.

In the second place, the quantity present in strong iso-agglutinating solutions of *Arbacia* is extraordinarily variable; thus on June 18, 1913, an agglutinating solution was made by addition of 3 parts sea-water to 2 parts cut-up ovaries and eggs of *Arbacia*. The test on *Nereis* sperm showed: (1) undiluted, formation of a solid ring-coagulum; (2) diluted one-half with sea-water, solid ring-coagulum; (3) diluted to one-fourth with sea-water, slight permanent coagulum; (4) diluted to one-eighth, small coagulated masses; (5) diluted to one-sixteenth, nearly neutral; (6) diluted to one-twenty-fifth, absolutely neutral. Acting on *Arbacia* sperm the same solution gave a 5-second reaction at 1/200 dilution. Other samples equally strong in the iso-agglutinating substance were nearly neutral for *Nereis* sperm undiluted.

In the third place it is possible to precipitate out all of the *Nereis*-active substance with *Nereis* sperm and leave the full complement of iso-agglutinating substance. Thus on June 19 an agglutinating solution was made by addition of 3 parts sea-

water to 2 parts ovaries and eggs of *Arbacia*; the fluid was filtered off. Undiluted it had a powerful effect on *Nereis* sperm but was negative at 1/16 dilution; 1 cc. was then placed in each of three tubes *A*, *B*, *C*. To *A* was added 2 drops of an aggregative *Nereis* sperm-suspension, to *B* 4 drops of the same, to *C* 8 drops of the same. Precipitates formed in all. On testing the supernatant fluids all were found to be negative to *Nereis* sperm. The hetero-agglutinating substance was completely fixed even in *A*. Tested for the iso-agglutinating substance, *A* was shown to be equal in power to the control. The tests gave:

- (A) 1/800—faintest reaction
- Control 1/800—faintest reaction
- (A) 1/400—12 second reaction
- Control—1/400—8 second reaction

Thus while all the *Nereis*-active substance was destroyed the iso-active substance remained in full amount.

This is of course conclusive; but I was also able to show that the sperm of *Ctenolabrus* (teleost) may precipitate the hetero-active substance leaving the iso-agglutinating substance intact. The hetero-active substance would therefore appear to be rather generally toxic to foreign sperm. It is present in greater quantity in the blood than in egg solutions as shown by definite experiments which need not be quoted in detail. It is rather probable, indeed, that its presence in the egg solutions is due to contamination by the blood. But I did not test this out fully, though it could readily be done by successive washings of the eggs.

A rather embarrassing fact that appeared in the course of the above tests is that *Nereis* sperm may destroy considerable quantities of the iso-agglutinating substance if present in excess of the amount required to fix the hetero-active substance. This came out first in the test of *C* (under June 19 above), which contained at least 4 times the amount of *Nereis* sperm needed to fix the hetero-active substance, for the supernatant fluid in this tube turned out to be several times less active in its iso-agglutinating properties than the untreated control. It looked as though the *Nereis* sperm would combine more actively with the iso-agglutinating substance than the *Arbacia* sperm itself.

There are, however, strong reasons against accepting this conclusion: (1) there is no visible effect of the iso-agglutinating substance on the Nereis sperm as should be the case if there were actual combination. (2) The action of the Nereis sperm is ferment-like, inasmuch as the iso-agglutinating substance continues to disappear progressively for a long period of time in the presence of excess of Nereis sperm, whereas this is not the case in the combination with Arbacia sperm. I shall therefore omit the details of the experiments under this head, being convinced that we are dealing here with a case of actual destruction of the iso-agglutinating substance and not with chemical combination.

5. SOME SPECIAL PROPERTIES OF THE FERTILIZIN

It would be interesting to know something concerning the chemical nature of this substance which is secreted by the eggs in such considerable quantities. This subject was, however, left for another occasion, as the investigation of its biological properties offered more problems than could be investigated at the time. Apart from these biological properties, all that I am now able to say about it is of a negative nature.

(a) It is colorless: Thus in table 2 all the washings after the first two were colorless though the concentration of the substance was considerable; similarly in table 3. (b) It must possess very considerable molecular size for it can be completely removed from any solution by filtering through a Berkefeld filter. Whether the solution be fresh or not, or of whatever concentration makes no difference; the filtrate obtained through a Berkefeld filter was invariably devoid of sperm agglutinating properties. On the other hand, it readily passes specially hardened filter paper. (c) Correspondingly it is non-dialyzable, not passing through the walls of celloidin tubes even in 24 hours. (d) It is extremely heat resistant being destroyed only slowly at the boiling point (see Study V, 1913). (e) As stated in my preliminary paper, I am indebted to Dr. Otto Glaser for the determination that it does not give the usual protein tests,* even in the most concentrated solutions obtainable (see Glaser, 1914a) It is

thus colorless, non-filterable, non-dialyzable, very thermally stable and apparently non-protein in its character.

The stability of the substance is also evidenced in its lasting qualities. Filtrates from egg-suspensions were kept and tested after various intervals of time. Thus the filtrate from a 33 per cent egg-suspension of June 21, was of 1600 agglutinating power; on August 26 it was of 100 agglutinating power. Similarly, the filtrate from a 33 per cent egg-suspension of June 20 was of 1600 agglutinating power; on August 26 it was of 100 agglutinating power. On the other hand, an agglutinating solution of June 30, of 800 agglutinating power was practically negative on August 26. In this tube there was more evidence of bacterial action than in the other two. Again, a distilled water extract kept from September, 1912, was of 16 agglutinating power on July 7, 1913.

In Study V, I also noted that its production is confined entirely to the eggs. It is not contained in the blood, and could not be extracted from other tissues.

6. ANTI-FERTILIZIN

We have noted the extreme avidity of the spermatozoa for the fertilizin produced by the eggs. Neither the blood nor tissues appears to contain any substance capable of uniting with the spermophile side-chain which causes agglutination of the spermatozoa, or of neutralizing its action. However, such a substance is contained within the egg itself, and its discovery is one of the most interesting points in the mechanism of fertilization, because it would appear from the facts considered in section 7 that it is the occupancy of the spermophile side-chain of the fertilizin by this substance, which I propose to call anti-fertilizin, that prevents polyspermy, or refertilization of eggs.

Its presence can be demonstrated in the test-tube by methods which extract the internal substance of the eggs. But as the jelly surrounding the eggs contains an immense excess of fertilizin, complete neutralization of sperm agglutinating power can be obtained only after the jelly is removed, and still better if the eggs have been repeatedly washed until their fertilizin production is greatly reduced.

To consider the latter method first: The matter was first brought clearly to my attention in the experiment listed in table 2. In the 34th washing the supernatant fluid tested to 1/20 dilution; it was poured off, leaving 1.5 cc. in the tube, and 10.5 cc. of sea-water was added. When the eggs were stirred up they at once disintegrated, liberating the red pigment so that the fluid which in previous washings had been colorless became bright red. When this fluid was tested with a fresh sperm suspension it was absolutely without agglutinating effect. The dilution of the 20 power fluid of the 34th washing was 8 times, so that even if the eggs had suddenly ceased the production of the agglutinating substance the fluid would have contained 2.5 agglutinating units and should have given at least a 10-second agglutination reaction; but it was absolutely negative. The agglutinating substance had therefore been neutralized by some substance escaping from the disintegrating eggs.

In the experiment listed in table 3, the repetition of this result was of course looked for. On the morning of August 24, after 35 washings on previous days, I noticed that pigment was beginning to diffuse from the eggs in tubes *A* and *B*, but it had not extended above the 1.5 cc. mark. The eggs were beginning to break down. Without disturbing them, I tested the colorless supernatant fluid and got undiluted an 8-second reaction in *A* and a 7-second reaction in *B*. Both tubes were then vigorously shaken until the eggs were disintegrated and the fluid colored with the escaping pigment. Tested in 4 minutes *A* was negative for agglutination, *B* gave a 4-second reaction. Tube *B* was again shaken and in 11 minutes more its agglutinating action had also disappeared.

There can, therefore, be no doubt that the egg contains in its interior a substance that unites with the spermophile group of the fertilizin.

In these experiments it was interesting to note that after the agglutinating action had disappeared, the solution was very strongly chemotactic, so that the drop injected into the sperm suspensions formed a ring of active, entirely unagglutinated spermatozoa. The chemotactic substance is certainly distinct

from the fertilizin.* The same observation was made also on other occasions.

The presence of the anti-fertilizin in the eggs could also be demonstrated in extracts of fresh eggs. These were made (1) by extracting in distilled water, (2) by drying eggs and grinding them in sea-water, (3) by grinding fresh eggs with sand in a mortar. I had previously often noticed that such extracts gave decidedly atypical agglutination of the spermatozoa, as compared with the action of the secretion of the eggs into sea-water. Distilled water extracts were then examined to find the cause for such atypical behavior. After it became clear from the experiments quoted above that the eggs contained the anti-fertilizin, the method of making the distilled water extracts was modified by first shaking the eggs as free from jelly as possible, so as to avoid the great excess of fertilizin contained in the jelly. These experiments yielded the following results:

August 22, 1913: 10:35 A.M. washed eggs were shaken six times in a test tube to get rid of the jelly. The eggs were washed twice again and shaken six times more; 60 per cent lost jelly; let stand. 2:15 P.M. Poured off sea-water to 2 cc. and added fresh sea-water to 8 cc. and divided equally in two test tubes, *A* and *B*; allowed to settle. 2:27 P.M. Poured off sea-water from settled eggs to 1 cc. in both *A* and *B*. To *A* added 7 cc. sea-water. To *B* added 7 cc. distilled water. The eggs were allowed to stand until they were completely plasmolyzed in *B* and the supernatant fluid was bright red. Tests were then made within an hour, or less, of the supernatant fluid in *A* and *B*, for the fertilizin, with somewhat surprising results:

(A) sea-water secretions Dilution tests	(B) distilled water extract Dilution tests
	1/1280—faint trace
	1/640 —7 sec. reaction
	1/320 —12 sec. reaction
1/160—negative	1/160 —12 sec. reaction
1/80 —negative	1/80 —12 sec. reaction
1/40 —negative	1/40 —10 sec. reaction
1/20 —6 sec. reaction	1/20 —14 sec. reaction (strong chemotaxis)
1/1 —90 sec. reaction	1/1 —12 sec. reaction (strong chemotaxis)

Thus if only extreme dilutions were compared the conclusion would be that *B* is 32 times stronger than *A*, whereas comparison of the undi-

luted fluid would lead to the conclusion that *A* was $7\frac{1}{2}$ times stronger than *B*. The fact that the time of the reaction of the *B* extract is about the same at all concentrations is in most pronounced contrast to the sea-water secretions in a very large number of tests, which had shown that the duration of the reaction is about proportional to the concentration of the agglutinating substance. The inference from the fact would be that the extract (*B*) contained an inhibitor for the agglutinating reaction which is diluted equally with the agglutinating substance. This might be a substance entering into combination with the agglutinating substance, but slowly and loosely at first.

4:07 P.M. Tube *B* was shaken vigorously to complete the plasmolysis. Tube *A* was stirred up. Both were then left until the next morning.

August 23, 1913: Tests of agglutinating power on fresh sperm:

<i>Tube A</i> <i>Dilution tests</i>	<i>Tube B</i> <i>Dilution tests</i>
1/20—6 sec.	Negative at all dilutions 1/1280,
1/10—12 sec.	1/640, 1/320, 1/160, 1/80, 1/40, 1/20,
1/1 —190 sec.	1/10, 1/1, but showing decided chemotaxis.

Thus the neutralization of the fertilizin had become complete in the intervening time.

The disappearance of the agglutinating substance is not due to any destructive effect of distilled water on it, for control experiments showed that this was not the case.

A similar experiment on August 20 gave even more clear cut results. In this case (1) the eggs were deprived of jelly by 6 shakes, and examination of the eggs in ink showed that jelly was almost completely removed. (2) The eggs were allowed to settle and the supernatant fluid poured off. (3) The eggs were then divided into two equal parts in test-tubes *A* and *B*. (4) To *A* added 9 cc. fresh sea-water, to *B* added 9 cc. distilled water. (5) Each was stirred up twice and stood 30 minutes.

Tested with fresh 1 per cent sperm suspension, *A* gave a 10 second reaction at 1/40 dilution. *B* was negative at 1/2 and gave a faint reaction only, undiluted. Thus as *B* must have contained as much fertilizin as *A*, its disappearance must have been due to escape of antifertilizin by plasmolysis, inasmuch as we know that distilled water does not destroy it.

It would follow from this experiment in which the jelly was practically entirely removed, that the eggs contained an amount of antifertilizin sufficient to neutralize all the fertilizin present in them.

A third experiment yielded results similar to the first:

August 23, 1913: Fine lot of fresh eggs; shaken 6 times, 2:15 P.M. in test tube. Examination in ink showed about half have entirely lost

jelly, and that it is much reduced in nearly all. About 2 per cent of the eggs broken.

2:33 P.M. washed: Divided in two equal lots in test-tubes *A* and *B*.
3:08 P.M.: After settling of eggs poured off sea-water down to 2 cc. in each tube. To *A* added 6 cc. sea-water. To *B* added 6 cc. distilled water. Stirred several times until *B* was thoroughly laked.

Tests with fresh 1 per cent sperm

A. 3.40 P.M.	B. 4.05 to 4.15 P.M.	B. 4.35 P.M.
	1/640— 6 sec. reaction	1/640— 6 sec. reaction
	1/320— 7 sec. reaction	1/320— 8 sec. reaction
	1/160— 10 sec. reaction	1/160—12 sec. reaction
1/80— 4 sec. reaction	1/80 — 36 sec. reaction	1/80 —25 sec. reaction (chemotaxis)
1/40— 8 sec. reaction	1/40 — 60 sec. reaction	1/40 —22 sec. reaction
1/20— 13 sec. reaction	1/20 —110 sec. reaction	1/20 —25 sec. reaction
1/10— 17 sec. reaction (1)	1/10 — 22 sec. reaction	1/10 —50 sec. reaction
1/1 —130 sec. reaction (1)	1/1 — 75 sec. reaction	1/1 —90 (?) sec. reaction

The second test of *B* indicated some progress in the neutralization of the fertilizin (cf. 1/20, 1/40, 1/80). The *B* dilution series is extremely irregular. Reading back from 1/1 (first test) the big jump from 1/10 to 1/20 indicates a breaking of the fertilizin X anti-fertilizin combination at this dilution.

August 24: Test showed some fertilizin present in *B*. Having poor sperm for indicator, left until next day. Shook up *B* vigorously; stirred *A*.

August 25: *B* now negative; *A* strongly agglutinative.

The distilled water extracts thus show the presence of a substance which combines quickly with the spermophile group of the fertilizin. In the experiment where the jelly was all removed by the preliminary shaking, there was practically immediate neutralization of the fertilizin. In the first and third, where the fertilizin was present in excess owing to the presence of some jelly, the excess was only slowly combined. Experiments with extracts of dried eggs (August 26) and with destruction of eggs by shaking or grinding also showed the presence of anti-fertilizin.

7. THE NECESSITY OF FERTILIZIN FOR FERTILIZATION

It will be apparent from the preceding facts and considerations that the fertilizin answers some of the requirements of a binding link between ovum and spermatozoon. When we consider the extraordinary activity of the unfertilized egg in its secretion, and the equally extraordinary avidity of the spermatozoa for it, one cannot escape the conviction that it must be a link in the normal fertilization process. When, moreover, one finds that the egg contains a more centrally located substance that can occupy the same combining group as the sperm, one seems to have, before one, a view of a mechanism for preventing polyspermy.

I adopted, then, the working hypothesis that this substance is necessary for fertilization and there followed immediately three corollaries, viz: (1) If it were possible to extract this substance from eggs, they should no longer be capable of fertilization; (2) fertilized eggs are incapable of uniting again with spermatozoa, hence, if the hypothesis is correct, they could no longer contain free fertilizin; (3) eggs in which membranes have been formed by methods of artificial parthenogenesis become incapable of fertilization; such eggs must also, therefore, be devoid of free fertilizin after they have reached the non-fertilizable condition if the hypothesis is correct. These consequences of the theory were actually found to be true.

a. Fertilization of washed eggs

The only way of which I could think of extracting the fertilizin from the eggs without injury, is the method of repeated washings. But we have seen (tables 2 and 3) that it is actually impossible to remove all the fertilizin from a mass of eggs by any number of washings, for the eggs continue to produce it until they go to pieces. However, the quantity produced in successive washings slowly diminishes. Therefore, we might expect on the basis of the theory, that there would be a gradual reduction of the percentages of eggs fertilized after many repeated washings. This actually occurs, as table 4 shows. It might also be anticipated that as

the quantity of fertilizin decreases, the developmental energy of the fertilized eggs might decrease, owing to relative incompleteness of fertilization. This also occurs. In table 3 (p. 536) it will be seen that fertilizations were made at the times of the 11th, 16th, 27th and 31st washings. In each case about three drops of both egg-suspensions *A* and *B* were taken, including several hundred eggs and fertilized with considerable excess of fresh sperm. It will be recalled that the eggs of lot *A* had been deprived of their jelly by shaking, whereas those of lot *B* retained the jelly. *A* lost its fertilizin relatively rapidly therefore, at first, but later the repeated washings caused removal of all the jelly in *B* also.

Table 4 shows the fertilization results, as regards the percentage of eggs segmenting (200 eggs were counted in each case). There is thus the anticipated decrease in percentage of fertilizations. Moreover, the time between insemination and the appearance of cleavage increases very much, and the eggs cease after a while to produce fertilization membranes and the cells tend to fall apart.

TABLE 4

DATE		NUMBER OF WASHINGS	
August 21	4:06 P.M. Fert. 1.	11	A 76.0% segmented B 97.5% segmented
August 22	9:13 A.M. Fert. 2.	16	A 76.0% segmented B 92.0% segmented
August 22	4:19 P.M. Fert. 3.	27	A 83.5% segmented B 97.5% segmented
August 23	9:13 A.M. Fert. 4.	31	A 39.0% segmented B 30.0% segmented
August 23	11:30 A.M. Fert. 5.	33	A 25.0% segmented B 24.0% segmented

Concomitantly, with these effects of the series of washings, the developmental energy becomes greatly reduced. This was very obvious from the second fertilization. On August 24 (48 hours after fertilization) a large quantity of living material was contained in the second *A* fertilization, but not one had even approximately pluteus structure. The most common form was a

stereoblastula. In the second *B* fertilization there were a few abnormal prismatic plutei, while the majority were gastrulae; some stereoblastulae. The third fertilization resulted in extremely abnormal ciliated types. The fourth and fifth did not proceed beyond abnormal cleavage stages.

The eggs have evidently lost something which affects their power of fertilization. Table 3 shows the measure of loss of the sperm-agglutinating substance, and justifies the general conclusion that this is a factor in the result. The loss of other substances may also combine in the decrease of fertilizing power, but of this we know nothing definite. As a matter of fact, fertilizing power is gradually lost with decrease of fertilizin content of the egg.

A considerable number of eggs go to pieces during the washings. This was especially noticeable in the *A* series of the above experiment. Thus after the 19th washing my notes record that eggs were stuck to the tube to the 3.5 cc. mark, whereas in *B* where the eggs were protected by jelly, only a very few stuck and these did not extend above the 1 cc. mark. Such eggs liberate antifertilizin, and if this is present in sufficient amount it may conceal the fertilizin production of the intact eggs. This phenomenon appeared a number of times in my earlier experiments when eggs were shaken too strongly in the effort to get rid of the jelly, resulting in injury to a large proportion of eggs, which soon broke down and neutralized the fertilizin produced by intact eggs.

One other experiment with apparently much more decisive results may be given (Aug. 18, 1913). In this experiment the shaking lasted 45 seconds and over 99 per cent of the eggs were stripped of jelly. The eggs were then precipitated by the centrifuge using just enough force to ensure complete precipitation, and the supernatant fluid poured off. It was very highly colored, showing that many eggs had been broken. It tested to a dilution of 1/12800, giving a 5 second reaction. The eggs were then repeatedly washed about 10/1.5 each time. The time of change and tests of supernatant fluids are shown in table 5, p. 552.

3:45 P.M. The fluid in 15 now gives a 6 second reaction, showing that some eggs are still producing fertilizin, but that it is rapidly neutralized.

After the 8th washing these eggs had extremely little capacity for fertilization, although only 24 hours old: Fertilization 1. 9:08 A.M. August 19: A few drops of stirred up egg suspension put in 9 cc. seawater and 3 drops 1 per cent sperm added. This is an enormous excess

TABLE 5

WASH- ING	TIME	TEST
2	2:48 P.M.	1/6400
3	3:15 P.M.	1/800
4	3:47 P.M.	1/100 (bulk of eggs 0.2 cc.)
5	4:05 P.M.	1/20
6	5:25 P.M.	1/10
7	Aug. 19: eggs had stood in tubes over night	
	8:37 A.M.	1/10 negative. 1/1—13 seconds
8	9:10 A.M.	1/10—11 seconds
9	9:42 A.M.	1/10—10 seconds
10	10:00 A.M.	1/10—(faint)
11	10:20 A.M.	1/1 —11 sec. (strong chemotaxis)
12	10:47 A.M.	1/1 — 9 sec. (strong chemotaxis)
13	11:08 A.M.	1/1 — 5 sec.
14	11:53 A.M.	1/1 — 4 sec.
15	2:00 A.M.	1/1 -- (negative)

of sperm. The eggs were rapidly literally covered with a single layer of adherent spermatozoa, but in spite of this only about 1 per cent divided (out of 200 eggs counted 3 were divided irregularly at 11:15). Some fresh eggs fertilized for control of sperm at same time showed practically all eggs divided at this time. Fertilization 2. 10:02 A.M. Same as 1, with control: 2:03 P.M. About 1 per cent divided; irregular, delayed; 80 per cent of control. Fertilization 3. 11:02. Same as 1, with control: 2:15 P.M. About 2 per cent divided; control 99 per cent divided.

Thus these eggs had almost entirely lost the capacity for fertilization with their loss of fertilizin, although they were only 24 hours in the sea-water. However, I regard the result given in the first experiment under this head as more typical for mere loss of fertilizin. In the experiment just quoted, the eggs had been injured to such an extent that they were liberating anti-fertilizin also, and I believe that this combined with the fertilizin in the cortex of the egg so as to block fertilization in this way. The extraordinarily rapid decrease of agglutinating power of successive washings (1, 12,800; 2, 6400; 3, 800; 4, 100; 5, 20; 6, 10; 7, 2 (?); 8, 10; 9, 10; 10, 10; 11, 1; 12, 1; 13, 1; 14, 1; 15, 0) is such as occurs only with injured eggs.

Other experiments give similar results. In one of these, the fertilizing power was reduced to about 1/8 in the third washing

after violent shaking which removed the jelly and in which the agglutinating power had been reduced from 400 to 10. It would appear from this that it makes no difference how rapidly we dispose of the fertilizin; the eggs are no longer capable of fertilization when it is gone.

b. Cessation of fertilizin production after fertilization

In a previous paper (Lillie '13) I noted that eggs of *Nereis* cease to produce sperm-agglutinating substance soon after fertilization, but that apparently this was not the case in *Arbacia*. I, however, ventured the suggestion that the apparent continued production of the sperm-agglutinating substance in *Arbacia* might be due to the presence of unfertilized eggs, of which there was a considerable percentage in the cultures. This may have had something to do with the results there described, but the factor that leads to an apparent continuation of fertilizin production in fertilized eggs is its presence in high concentration in the jelly, a condition that I, by no means, realized at first. When this factor was eliminated it was easy to demonstrate that fertilized eggs no longer produce the substance. The crucial experiments were as follows:

Experiment 1: August 23, 1913: A large quantity of unusually fine eggs were washed three times and fertilized at 2:30 P.M. with considerable excess of sperm. At 2:40 P.M. microscopical observation showed that nearly all had beautifully formed fertilization membranes. About 10 per cent of the eggs were without jelly (observation in ink). These usually had as fine membranes as the others.

The eggs were divided in two equal quantities in test tubes *A* and *B*. 2:50 P.M.: *B* was shaken 6 times to get rid of the jelly. Examination in ink showed that the jelly was removed completely from practically all the eggs. *A* (with jelly left on eggs) and *B* were then submitted to a series of washings with tests for fertilizin as shown in table 6, p. 554.

Thus in five washings the *B* fertilized eggs without jelly no longer gave the fertilizin test. This number of washings would be only barely sufficient to remove the agglutinating substance present before fertilization and the loose jelly. Two successive negative tests were given, showing that the production of the agglutinating substance has ceased absolutely. The *A* eggs were

TABLE 6

3:10 P.M.	A 1	Washed 8/2.0	
	B 1	Washed 8/1.0	
3:25 P.M.	A 2	Washed 8/1.5	
	B 2	Washed 8/1.0	
3:43 P.M.	A 3	Washed 8/2.5	
	B 3	Washed 8/1.0	Test 1/1—22 seconds
4:08 P.M.	A 4	Washed 8/2.0	
	B 4	Washed 8/1 0	Test 1/1— 9 seconds
4:53 P.M.	A 5	Washed 8/1.5	Test 1/1 over 75 seconds
	B 5	Washed 8/1.0	Test 1/1—negative
4 58 P.M.	A 5		Second test 1/1—60 seconds
	B 5		Second test 1/1—negative

apparently still producing it vigorously, but that is only because they carried the fertilizin-saturated jelly with them. Ninety-eight per cent of the *B* eggs segmented perfectly.

The result is in accord with the assumption that all free fertilizin is fixed *at the moment of fertilization*, or membrane formation. But I know of no way of absolutely demonstrating that the fixation takes place thus rapidly. But if such fixation is the mechanism for prevention of polyspermy, as I believe, the fixation of all free fertilizin must take place in a few seconds at most.

A duplicate experiment was made on August 25. The methods of Experiment 1 were repeated exactly. The eggs were not, however, so good a lot and the membranes did not stand put so far from the eggs. The shaking of lot *B* removed the jelly from most, but not from all. In this experiment tube *B* came negative on the sixth and seventh washings; whereas tube *A* gave a 90-second reaction, undiluted, on the sixth washing. Over 98 per cent of the eggs segmented and the unsegmented eggs had spindles.

Both experiments were clear-cut; there can be no question that after fertilization the eggs cease to produce fertilizin. Two effects are involved; the disappearance of fertilizin, and failure to produce more. The first effect might be due to extrusion of all fertilizin from the eggs; but it seems improbable that this could take place with sufficient rapidity to prove effective as a mechanism for preventing polyspermy, and inasmuch as we know that the eggs contain anti-fertilizin capable of occupying the spermo-

phile group of the fertilizin, it is more reasonable to conclude that the disappearance of sperm-agglutinating power from the washings of fertilized eggs deprived of jelly is due to neutralization of the substance remaining in the egg. In this connection it is instructive to note that eggs commonly lose pigment on insemination; I always have found the anti-fertilizin accompanied by pigment; it is, therefore, natural to suppose that anti-fertilizin is carried to the surface of the egg on insemination, thus affording the opportunity for combination with the fertilizin and its neutralization.

The other possibility that the sperm combines with all the free fertilizin in the egg appears to me inadmissible, because (1) the surface of the egg is relatively so enormous, and (2) the amount of fertilizin which the sperm is capable of binding is very small relative to the amount produced by the eggs even at a single washing (cf. sec. 3, p. 538). The bulk of the sperm actually used for insemination is insignificant compared to the bulk of the eggs, and only a small proportion of the sperm used actually fertilizes so that the amount fixed by the sperm itself is excessively minute compared to the quantity contained in the egg.

We have seen (p. 529) that 1 cc. of eggs charges 5 cc. of seawater to 1600 units. We have also seen that such eggs contain or produce many times this quantity in successive washings; and that it takes 1 cc. of 3 per cent sperm to fix 1 cc. of 64 power agglutinating solution. Thus it would require $1 \text{ cc.} \times 1600/64 \times 5$ or 125 cc. of 3 per cent sperm to neutralize the agglutinating substance produced by one washing of 1 cc. of eggs in 5 cc. of seawater. But a single drop of 3 per cent sperm would be much more than sufficient to fertilize the same amount of eggs. The disproportion is even greater than indicated in this rough calculation, which is, however, sufficient to show the absurdity of explaining neutralization of the agglutinating substance of the fertilized eggs by the sperm used in fertilization.

Theories of prevention of polyspermy, or of the non-fertilizable character of eggs already fertilized, proceed along two lines: (1) that the membrane formed on fertilization is impermeable to spermatozoa, (2) that the protoplasm of the fertilized eggs has

undergone some 'physiological' change which prevents union with the sperm. The first theory is an old one based on conditions in the sea-urchins; it is inapplicable to the ova of most animals, which do not form special membranes after fertilization; whatever value the fertilization membrane may have in special cases as guarding against polyspermy, it has been shown for some cases at least that the real cause of failure of refertilization is more deeply seated. Thus Wilson ('03) shows for *Cerebratulus* that after the germinal vesicle has faded, cytoplasmic fragments devoid of nucleus may be fertilized and undergo typical merogonic development; but enucleated fragments of fertilized eggs are incapable of refertilization: "Even when such fragments are placed in water containing sperm immediately after the section before they have assumed a spherical form, they fail to fertilize, though spermatozoa may be observed adhering to their periphery." Wilson rightly attributes the sterility of such fragments to a physiological change of the cytoplasm.

The theory of a physiological change of the cytoplasm following fertilization has been generally accepted, but there has been no idea of the nature of the change involved. Wilson directs attention to the similar sterility of egg fragments prior to fading of the germinal vesicle and suggests that the cytoplasmic change following fertilization is physiologically the opposite of that following the fading of the germinal vesicle. Now, as I have shown the probability that the agglutinating substance is not present in an active form in *Arbacia* prior to fading of the germinal vesicle, and that it becomes neutralized by union with anti-fertilizin following fertilization, it is clear that this mechanism answers the requirements of the problem. The fundamental mechanism for the prevention of polyspermy is the neutralization of the fertilizin by the anti-fertilizin present in the egg; i.e., the occupancy of the spermophile side-chain of the fertilizin by the anti-fertilizin.³

³ Note: Delage ('01) determined for *Asterias glacialis* that fragments of the ovocyte with intact germinal vesicle are sterile, so that merogony cannot be produced. As soon as the germinal vesicle fades fragments are fertilizable and merogonic development may follow. Wilson's observations are in accord with

The question may be raised why such neutralization of the fertilizin is delayed until the moment of fertilization? The answer to this difficulty is fairly clear. The fertilizin is located in the cortex of the egg, and the anti-fertilizin is more deeply situated; they therefore do not interact so long as the cell body as a whole is quiescent. But as soon as the cortical fertilizin becomes activated by union with the sperm it at once begins to attack certain substances in the egg, as demonstrated in the third part of this paper; this sets up diffusion evidenced by escape of pigment, and by cytoplasmic flowing, and the two substances are brought together and interact. While this explanation is partly hypothetical, the spatial separation of the fertilizin and anti-fertilizin and the quiescent character of the cell-body in the unfertilized egg are facts; so also are the movements of diffusion and the cytoplasmic currents set up on fertilization. However it is possible that some other factor is operative in the interaction of fertilizin and anti-fertilizin following fertilization, which has entirely escaped attention.

c. The cessation of fertilizin production after formation of membranes by butyric acid

Some methods of artificial parthenogenesis cause eggs to become non-fertilizable; others apparently do not. In the sea-urchins it would seem that those methods that cause membrane formation result in the non-fertilizable condition while those which do not, need not have such an effect. Thus the use of hypertonic sea-water causes a certain amount of parthenogenesis

this as noted. But Delage farther asserts that a second change occurs after the formation of the second polar globule so that non-nucleated fragments again become sterile, although entire eggs can still be fertilized. This result is unsupported so far as I know, and would be difficult to explain on the basis of the mechanism described in this paper except on the assumption that the original contribution of nuclear sap necessary for the production of fertilizin has become exhausted, and that presence of the nucleus had become necessary for continued production of fertilizin. Delage shows that during the fertilizable period of the cytoplasm when merogony can be effected parthenogenetic agents are especially effective, a result that agrees particularly well with my point of view.

in sea-urchin eggs without membrane formation but the development tends to be quite abnormal. Loeb has observed that membrane formation may be induced in such eggs after they have reached even the 8 or 16-celled stage by addition of sperm; they are still fertilizable, so far at least as the cortical changes are concerned. It is fair to assume in such a case that the lack of membrane formation is evidence of persistence of free fertilizin in the cortex, for the only cases in which I found this substance absent from mature eggs are eggs with membranes formed. This case, therefore, need offer no difficulty for the contention that the fertilizin is necessary for fertilization.

On the other hand, Loeb's method of treatment with fatty acids leads to membrane formation and such eggs are non-fertilizable, as Loeb states and I have myself observed. Loeb, however, goes on to assert that if such eggs are shaken so as to destroy the membrane and if sperm is then added, a new membrane is formed and the typical development of sperm-fertilized eggs follows. This would seem at first sight to run counter to my theory unless it could be shown that membrane-formation by butyric acid differed from membrane formation by sperm in not causing cessation of fertilizin production. However, as I shall show immediately, the production of fertilizin does cease after the formation of good membranes by butyric acid.

It is necessary, therefore, to examine Loeb's statement rather carefully. It is as follows:

Dr. Kupelwieser und ich stellten diesen Versuch an, indem wir die Membranbildung bei unbefruchteten Eiern mittels Buttersäure hervorriefen, diese Membran dann *unmittelbar nach Bildung derselben*, durch Schütteln der Eier zum Platzen brachten und *sofort* Samen zufügten. Die Eier bildeten eine neue *dem Zytoplasma dicht anliegende Membran* und furchten sich mit einer der Samenbefruchtung entsprechenden Geschwindigkeit, und Zwar zunächst ausnahmslos in zwei Zellen. (Loeb '09, p. 210; italics mine).

It is implied that such superposition of fertilization on parthenogenesis would not take place unless the experiment was performed immediately after membrane formation. Presumably some fertilizin remained unbound for a short time and on account

of its relatively small amount the new membrane was formed close to the egg. The implied fact that the experiment failed after a short time supports my theory of the function of fertilizin, and the stated result is not at all inconsistent with my views. According to my point of view the possibility of superimposing fertilization on parthenogenesis would persist so long as a sufficient quantity of fertilizin remained unbound, i.e., so long as the reaction was incomplete.⁴

The non-fertilizable character of such eggs implies on my theory an absence of free fertilizin. This condition obtains, as the following experiments show:

Experiment August 26, 1913: A quantity of fresh Arbacia eggs were taken and some were set aside in a test tube as control (0.14 cc. when settled). The balance were concentrated in 3.4 cc. sea-water.

3:47 P.M. The latter were then added to 25 cc. butyric acid in sea-water made by adding 2.8 cc. $\frac{N}{10}$ butyric acid to 50 cc. sea-water.

3:47 1/2 P.M. Half of the eggs with acid were poured into 1500 cc. sea-water in crystallization dish *A* and stirred up to stop the action of the acid.

3:48 P.M. The remainder were poured into 1500 cc. sea-water in crystallization dish *B* and stirred up. *B* was over-exposed and the eggs agglutinated heavily. In *A* there was no agglutination and nearly all of the eggs formed fine membranes. The jelly was absolutely all gone owing to the action of the butyric acid, so there was no need to shake them.

The eggs in *A* and *B* were then concentrated, and placed in graduated tubes similar to the control. When settled there was 0.3 cc. eggs in each, estimated to be at least 4 times as many as in the control tube when allowance is made for the bulk of the jelly in the control. All were then submitted to successive washings with tests as shown in table 7, p. 560.

Thus the *A* eggs, with membranes formed, came negative in four washings, at least as rapidly as fertilized eggs, whereas the unfertilized control with fewer eggs was still showing an agglutination strength of about 10 (control 4, 1/1—50 seconds). There

⁴ The essential problem is whether fertilization is of the nature of an irreversible chemical reaction, as I claim, or is merely a physical surface effect. Although this question has been investigated a good deal, it is still far from being definitively settled. The students of artificial parthenogenesis take the latter point of view generally.

TABLE 7

	WASHINGS	TESTS FOR FERTILIZIN
4:13 P.M.		
Control 1	10/1.2	
4:30 P.M.	A 1 12/2.4	Test 1/10—11 seconds
	B 1 12/1.5	
Control 2	11/2.0	Test 1/1 —2 plus min.
4:42 P.M.	A 2 13/2.0	Test 1/1 —32 seconds
	B 2 13/1.5	Test 1/1 —45 seconds
4:50 P.M.		
Control 3	10/1.0	No test
4:55 P.M.	A 3 13/2.0	Test 1/1 — 7 seconds
	B 3 13/2.0	Test 1/1 —11 seconds
5:05 P.M.		
Control 4	10/1.0	Test 1/1 —50 seconds
5:08 P.M.	A 4 12/2.0	Test 1/1 negative
5:13 P.M.	B 4 12/1.5	Test 1/1 — 9 seconds
Later	A 5 about the same	Test 1/1 —negative
	B 5 as above	Test 1/1 — 8 seconds

is no doubt, therefore, that in eggs with membranes formed by butyric acid the agglutinating substance is fixed, as it is in fertilized eggs. Presumably, the same would hold true for other parthenogenetic methods which render the eggs incapable of fertilization.

The next day the washings were continued. On the sixth, washing *A* again showed a trace of the agglutinating substance, but came negative again on the remaining three washings. The reappearance is to be attributed to the few eggs without membranes which had been active all night, accumulating in this time sufficient agglutinating substance to show in the test though not producing sufficient to show in repeated washings. The *B* eggs came negative in the ninth washing, while the control eggs still gave a 40-second reaction undiluted.

The *B* eggs although exposed longer to the butyric acid did not form membranes, and they continued to produce fertilizin longer than the *A* eggs but in much less quantity than the control eggs. It might therefore, be expected that they would retain capacity for fertilization longer. The fertilization capacities were not

worked out in this experiment; however, Herbst ('00) found in his experiments on superposition of fertilization on parthenogenesis that eggs overexposed to butyric acid could be fertilized, though incompletely.

Ninety-five per cent of the *A* eggs showed no evidence of cytolysis on the next day, beyond the membrane formation. They were intact and healthy looking. The remaining 5 per cent were fragmented, or segmented, into about 16 to 32 cells, within the membrane and appeared cytolized. In the *B* eggs the agglutination had disappeared, and there was about the same percentage of dividing eggs as in *A*, but in absence of a membrane the cells were breaking apart.

A similar experiment on August 25 gave the same result, viz., disappearance of the agglutinating substance after membrane formation by butyric acid.

Summarizing this section, then, we may say that eggs are rendered incapable of fertilization by methods that eliminate the sperm-agglutinating substance whether (a) the substance is lost, as in some eggs after repeated washings, or (b) fixed by previous fertilization, or (c) fixed by parthenogenetic methods. The conclusion is therefore justified that the agglutinating substance is necessary for fertilization.

8. SUMMARY OF PART II: SPERMOPHILE GROUP OF THE FERTILIZIN

After considering a method for determining the quantities of fertilizin produced by ova of *Arbacia*, we demonstrated (1) that unfertilized eggs in sea-water produce this substance in extremely large quantities. The secretion begins presumably in the ovary with breaking of the germinal vesicle. Although the jelly is saturated with it and liberates it by solution in the sea-water, eggs without jelly continue to secrete if for three days, at least, during repeated washings, or as long as they remain alive. (2) The spermatozoa of *Arbacia* possess extreme avidity for the fertilizin and bind it in definite amounts. In the case of fresh active sperm the binding is evidenced by reversible agglutination of the spermatozoa. But a stale sperm suspension may also bind it, although

the agglutination does not take place, except in high concentrations. (3) The fertilizin of *Arbacia* eggs does not combine with *Nereis* sperm. (4) A substance, which I have named anti-fertilizin, may be extracted from *Arbacia* eggs, by breaking them up or by extracting them in distilled water, which has the property of combining with the spermophile group of the fertilizin. It is present in sufficient amount to neutralize all the fertilizin contained in the eggs, excluding the jelly. (5) When the fertilizin is extracted from the eggs by repeated washings, they are no longer capable of fertilization. (6) Fertilized eggs produce no more fertilizin; that present at the moment of fertilization is neutralized by anti-fertilizin. (7) Eggs with membranes formed by butyric acid become incapable of fertilization, and they contain no free fertilizin.

III. THE OVOPHILE SIDE-CHAIN

INTRODUCTION

Having shown then, that the union of the agglutinating substance with the spermatozoon enters in some significant way into the process of fertilization, the problem was to ascertain in what way. The simplest idea (*viz.*, that the union is itself the fertilization process) was soon shown to be untenable, for the reason that the perivisceral fluid (blood) of the sea-urchin especially of ripe males and females, often contains a substance which absolutely inhibits fertilization in the presence of any quantity of sperm, but which has no inhibiting effect at all upon the sperm-agglutinating reaction. It does not enter into combination with the spermophile side-chain. In other words, the binding of the agglutinating substance by the sperm may be complete, but in the presence of an inhibitor contained in the blood none of the usual effects of insemination, no matter how heavy, follow.

The next suggestion was fairly obvious, *viz.*, that the substance which we have been calling the agglutinating substance on account of its effect on the spermatozoa, in reality possesses two side-chains active in fertilization, *viz.*, a spermophile and an ovophile side-chain; the binding of the sperm activates the ovo-

phile combining group of the fertilizin which then seizes upon egg receptors, and it is the latter union which results in membrane formation. If this were so, it is obvious that the spermatozoon is only secondarily a fertilizing agent, in the sense of initiating development, and that the egg is in reality self-fertilizing, an idea that agrees well with the facts of parthenogenesis and with the amazing multiplicity of means by which parthenogenesis may be effected. For the agents need only facilitate the union of the fertilizin and egg receptor.

The inhibiting action of the blood, from this point of view, is a deviation effect due to occupancy of the ovophile group of the fertilizin, either because the inhibitor in the blood is an antibody to the fertilizin or because it possesses the same combining group as the egg receptor. In such a case, the ovophile group of the fertilizin being already occupied by the inhibitor, fertilization could not take place.

This theory transfers the fertilizing power from some hypothetical substance contained in the spermatozoon to a definite substance contained in the egg itself, in relation to which the sperm acts merely as an activator. It does not necessarily predicate the precise mode of action of the fertilizing substance although the assumption is here made that chemical union with certain molecules in the egg, called egg receptors, is a necessary part of its action.

In the present section we shall proceed to examine the evidence for the existence of an ovophile as well as a spermophile combining group of the fertilizin.

1. THE INHIBITING EFFECT OF BLOOD OF THE SEA-URCHIN UPON FERTILIZATION OF EGGS OF THE SAME SPECIES

It is a common practice of embryologists to wash the eggs of marine invertebrates, which they propose to fertilize, twice or several times with sea-water, because it is a matter of experience that this procedure very greatly increases the proportion of eggs that fertilize. However, I know of no study of this really very striking phenomenon. The eggs are usually obtained for fertili-

zation by opening the animal and removing the ovaries so that the eggs are contaminated by blood or tissue secretions, and it is evident that the latter in some way inhibit the fertilization reaction, though they could hardly be supposed to have any direct injurious effect upon either sex element.

The necessity of washing the eggs carefully before fertilization, had been forced upon my attention by the poor results of certain experiments; and after some of the results recorded in the first part of the paper had been obtained, the phenomenon appeared to possess great significance, because the failure to fertilize in the case of unwashed eggs was clearly due to the presence of blood of the species. Some element of the blood must block the mechanism of fertilization, and it was clear that the analysis of the phenomenon must aid in understanding the mechanism.

The first experiments were undertaken to ascertain the extent of the inhibiting action of the blood of the sea-urchin. The nature of the effect may be seen from the protocol of the following experiment:

July 7, 1913: The blood of several males and females was obtained by cutting open the shell near the oral membrane and pouring out the perivisceral fluid (blood) in a finger-bowl. After the usual loose clot was formed the plasma was filtered through a soft filter-paper. The eggs used in the experiment came from one female and were washed in sea-water. The experiment was then set up as follows:

- 1 Quantity of eggs x plus 25 cc. sea-water plus 4 drops 1 per cent sperm
- 2 Quantity of eggs x plus 12.5 cc. filtered blood plus 12.5 cc. sea-water plus 4 drops 1 per cent sperm
- 3 Quantity of eggs $2/5$ X plus 10 cc. filtered blood plus 4 drops 1 per cent sperm

The fertilizations were made about the same time and the result was that in 1 practically all divided, in 2 only a fraction of 1 per cent divided, in 3 none divided. Moreover in 2 and 3 no fertilization membranes were formed in the undivided eggs and the egg nucleus remained intact; no spindles were formed. And this in spite of the fact that the spermatozoa were very active, so abundant that they formed 'halos' around the eggs by penetrating the jelly, and many reached the surfaces of the eggs.

This experiment was followed by a considerable number carried out in more detail. Four of these experiments are tabulated in table 8. The left-hand column gives the percentage of blood in

the sea-water in which the fertilizations were made. The other vertical columns give the percentages of eggs segmented for each concentration of blood on the same horizontal level. The last reading in each column gives the control, i.e., fertilizations made in sea-water alone with the same amounts of the same eggs and sperm used in the blood solutions. The eggs in the blood solutions were washed in the same way as the controls prior to insemination.

TABLE 8

	JULY 8: MIXED BLOOD OF MALES AND FEMALES	JULY 15: BLOOD OF MALES ALONE	JULY 22: BLOOD OF MALES ALONE
Blood serum in sea-water	Segmented eggs		
		Exp. 1	Exp. 2
1 per cent	75 per cent	90 per cent	90 per cent
3 per cent		25 per cent	60 per cent
5 per cent	10 per cent	10 per cent	30 per cent
7 per cent		1 per cent	30 per cent
9 per cent		25 per cent	45 per cent
10 per cent	about 0.2 per cent	50 per cent	
11 per cent			20 per cent
13 per cent			20 per cent
15 per cent			1.5 per cent
20 per cent	about 0.2 per cent		
40 per cent	about 0.2 per cent		
100 per cent			
Control: same eggs in sea- water.....	75 per cent	99 per cent	95 per cent

It will be noticed that in all these cases there is a marked reduction in the percentage of fertilizations when 5 per cent blood is present in the sea-water, greater in some cases than in others, which leads to the conclusion that the inhibitor is present in higher concentrations in some samples of blood than in others. This relation is dealt with below. In Experiments 1 and 2 of July 15, and in the experiment of July 22, there will be noticed after the first fall* with increasing blood-concentration a rise and second fall in the percentages of fertilizations as measured by cleavage of the eggs. This is unquestionably of some significance, but I have been unable to ascertain what its meaning may be. The same phenomenon occurred in later experiments.

While the experiments cited give effects only of mixed blood from the two sexes, and the blood of the males alone, other experiments, not so complete in all details, showed that females contain the inhibitor in their blood at least to the same degree as the males. If there is any sexual difference it is in favor of the females. The blood of the males was used in many tests alone because there was danger, in opening the females, of wounding the ovaries and so getting fertilizin into the blood, which as shown beyond, greatly modifies the results. There was of course the danger in using blood from males of getting spermatozoa from wounded testes, but as the eggs were in any case to be fertilized the result could hardly be affected by this; some control experiments showed that this was a rare occurrence in any event.

Individual variability in the amount of the inhibitor present in the blood being indicated in these experiments, a special study was made of this factor in order to test its extent, and if possible to correlate it with the condition of the animals. The individual tests were as follows (table 9): the columns give the percentage of eggs segmented after 2 to 3 hours. All the fertilizations of July 28 were made with a single sperm suspension and a single lot of eggs; similarly for each succeeding date. The various numbers therefore act as controls on one another. In all, a great excess of sperm was used. The counts were carefully made and checked by my assistant, Miss Brockett.

These tests of bloods of individuals bring out very extreme individual differences in the content of fertilization inhibitor in the blood, ranging in fact all the way from no inhibition to absolute inhibition of fertilization. But it is not easy to correlate the variations with the conditions of the individuals. The general hypothesis that I have been led to adopt, provisionally at least, is that the quantity of the inhibitor in any individual blood is related to the size and degree of maturity of the gonads. It would seem to be a very simple matter to determine whether this is or is not the case. However, in the first place, after the beginning of the season there are practically no animals with immature gonads to test, and in the second place, if the hypothesis were correct, it would be impossible to know in advance how long the

TABLE 9

MALES—20% BLOOD		FEMALES—20% BLOOD		FEMALES—100% BLOOD
July 28				
1	65 per cent	1	75 per cent	11 per cent
2	69 per cent	2	93 per cent	84 per cent
3	71 per cent	3	72 per cent	11 per cent
4	72 per cent	4	45 per cent	1 per cent
5	85 per cent	5	85 per cent	84 per cent
6	82 per cent	6	87 per cent	63 per cent
7	27 per cent	7	89 per cent	81 per cent
8	84 per cent	8	52 per cent	0
		9	80 per cent	24 per cent
		10	92 per cent	93 per cent
		11	6 per cent	2 per cent
		12		55 per cent
		13		0
		14		most
		15		over 90 per cent (rough estimate)
MALES—100% BLOOD		FEMALES—100% BLOOD		
July 29				
9	100 per cent	16		77 per cent
10	85 per cent	17		39 per cent
11	56 per cent	18		100 per cent
12	87 per cent	19		0
13	43 per cent	20		100 per cent
Control 97% (fertilized in sea-water)				
July 30				
14	2 per cent	21		35 per cent
15	25 per cent	22		100 per cent
16	29 per cent	23		15 per cent
17	93 per cent	24		65 per cent
18	93 per cent	25		19 per cent
19	100 per cent	26		99 per cent
		27		100 per cent
Control 100 per cent (fertilized in sea-water)				
July 31				
20	96 per cent	28		0
21	2 per cent	29		1 per cent
22	74 per cent	30		0
		31		38 per cent
		32		1 per cent
		33		41 per cent
		34		41 per cent
Control 94.5% (fertilized in sea-water)		35		89 per cent
		36		34 per cent

inhibitor might remain in the blood after the gonads were emptied. Thus the mere fact that an individual with small gonads had much inhibitor in its blood would not prove that the gonads had little to do with the formation of the inhibitor, for there would be no way of telling whether the reduction of the gonads was recent or not.

The considerations that led to the adoption of the above hypothesis are (1) that the condition of the gonads is the most variable thing in the summer sea-urchins, which coincides well with the variability of the amount of the inhibitor; (2) in general, the fluctuation through the season of periods of maturity of the sea-urchins coincides very well with the fluctuations in the amount of the inhibitor. Thus the animals were in very fine breeding condition during the first three weeks of July when the first tests showing abundance of the inhibitor in the blood, were made. They were in very poor condition then for about 10 days up to about August 2, when the individual tests were made showing such surprising decrease of the inhibitor. On August 6 when the material became better again, the inhibiting effect of the blood was stronger once more. (3) Although it was difficult, as said, in the individual tests to correlate the amount of the inhibitor with the condition of the animals, yet especially in the case of the females, those with large gonads appeared to have a large amount of inhibitor present. Thus females 28, 29, 30 and 32 were all ripe females that shed eggs when inverted after opening, in a watch crystal, whereas females 31, 33, 34, 35 and 36 were all individuals with medium size ovaries that shed no eggs when similarly inverted. Female 8, on the other hand, had small ovaries, shed no eggs, and yet possessed much inhibitor in its blood.

The conditions that determine the amount of inhibitor in the blood are thus a matter for more careful investigation. It may be possible to regulate the quantity experimentally by injection of fertilizin or of entire eggs. This merely indicates a possible method of approaching the problem.

I spent a great deal of time in endeavoring to meet the possible objection that the inhibiting action of the blood might be due to

contamination with the poisonous secretions of the epidermis, especially of pedicellariae. It seemed at first that this objection might be valid, for the period of my tests coincided with the season of poor material when the blood was relatively free from inhibitor. During this period, instead of following my earlier practice of opening the animal rapidly and pouring out the blood, I very carefully cut within the leathery peristome, avoiding all possible contamination with epidermal secretions and removed the blood with pipettes. This blood showed as a matter of fact little inhibiting effect. However, with the return of animals in good breeding condition, it was easy to show that the inhibitor was contained in the blood itself; and not only this, but also that epidermal secretions so far from increasing the inhibiting action of the blood, actually decreased it, a matter to which we return beyond.

The next question was whether the inhibitor acted on the egg alone, on the sperm alone, or merely by intervening in the reaction between the two? In the experiments undertaken to answer this question it was possible to show that the inhibitor certainly does not act strongly on either the egg or the spermatozoa alone. It is possible to collect eggs or sperm in filtered blood known to have a strong inhibiting action, and, after considerable exposure, to restore by washing nearly full fertilizing power, at least, to both kinds of sexual elements.

July 8: The blood used in this experiment allowed only 1 per cent fertilization when diluted to 5 per cent with sea-water; it therefore contained a great deal of the inhibitor.

1. As regards the effect on eggs alone: 9:41 A.M. The eggs of one female were placed direct from the ovary in some of this filtered blood. Transfers of two drops of eggs to 10 cc. of sea-water plus one drop 1 per cent sperm were made as follows: a. 9:42, b. 9:50, c. 9:55, d. 10:03, e. 10:23. About 60 per cent of each lot segmented. The blood carried over would be about 0.625 per cent. Thus the inhibiting effect was removed to a very great extent by washing. 2. In the case of sperm collected in the same blood and used for fertilization at 1 minute, 12 minutes, 22 minutes, and 37 minutes, no indication of inhibition of fertilizing power as compared with an identical series of controls was observed.

July 17: After 5 minutes' exposure to male blood sperm fertilized 20 per cent of the eggs; control sperm 90 per cent. (sperm = 0.004 per cent).

July 18: Sperm suspensions in two lots of blood, (a) from males, (b) from females. (a) Fertilized 40 per cent in 5 minutes and 25 per cent in 18 minutes, (b) fertilized 30 per cent in 5 minutes and 20 per cent in 18 minutes. (Control sperm fertilized 70 per cent each time. (Sperm = 0.0026 per cent)

July 19: Sperm suspensions in two lots of blood, (a) from males, (b) from females. (a) Fertilized 14 per cent in 10 minutes and 18 per cent in 30 minutes; (b) fertilized 23 per cent in 10 minutes and 20 per cent in 30 minutes. Control sperm fertilized 86 per cent in 10 minutes and 76 per cent in 30 minutes. (Sperm = 0.012 per cent).

July 21: Sperm suspensions in blood of males. In this case the sperm fertilized perfectly in two fertilizations. Sperm = 0.007 per cent in first and 0.0068 per cent in second. The blood contained a large amount of inhibitor as only 0.5 per cent segmented in it with 0.6 per cent sperm suspension.

August 1: A very detailed experiment showed that a specimen of blood very powerful in inhibitor had but little effect on the fertilizing power either of eggs or of sperm after washing.

On the whole we must conclude that though exposure to blood containing inhibitor may decrease the fertilizing power of sperm or of eggs, its effect is secondary and can probably be entirely removed by sufficient washing or overcome by sufficient concentration of sperm.

2. MODE OF ACTION OF THE INHIBITOR

It was natural to suppose that the inhibitor acted by occupying the side-chain of the fertilizin with which the sperm unites normally. If this were the case it must be possible to neutralize the agglutinating action of the fertilizin by a sufficient quantity of blood containing the inhibitor. Therefore, if we took two equal amounts of an agglutinating solution and diluted the one with sea-water and the other with blood containing the inhibitor, the agglutinating action ought to disappear more rapidly in the latter than in the former. This, however, is not the case, as the following experiment will show.

July 11, 1913: A given agglutinating solution was tested in the usual way by diluting with sea-water and found to be of 400 agglutinating power. Part of it was then diluted to 1/100 with blood; on test with sea-water sperm suspension it gave two tests of 22 seconds each. Diluted to 1/400 with blood, it gave a 10 second reaction with a sea-water sperm suspension, and an 11 second reaction with a blood sperm suspension.

The same result was obtained in other experiments and I could find no evidence that blood decreased the delicacy of the agglutinating reaction.

If then the fertilizin is essential for fertilization, and if the inhibitor in the blood does not operate by preventing union of this substance with the sperm, it follows that another side-chain of the fertilizin capable of being occupied by the inhibitor is also operative in fertilization. There must be action of the fertilizin on the egg as well as on the sperm, and hence we may assume an ovophile as well as a spermophile side-chain of the fertilizin, and the inhibitor must act by occupying the former.

Now Robertson ('12) has stated that various proteins when added to the sea-water can be shown to inhibit membrane formation by sperm,*though as a matter of fact his experiments demonstrate such a result only for ovomucoid in concentrations above 0.25 per cent. It might therefore be supposed that the inhibiting effect of blood of the same species was only a special case of protein inhibition. There are, however, considerations that show such a view to be untenable. (1) As already shown the degree of inhibition by undiluted blood ranges all the way from zero to 100 per cent; and we cannot suppose that the amount of protein in the blood of different individuals varies to the extent required to explain such an extraordinary difference. (2) In the second place the mode of neutralizing the inhibitor, as shown below, involves an enormous increase of colloid content of the blood, for it consists in saturating the blood with fertilizin and dissolved jelly; the blood may thus become actually syrupy in consistency and yet have no inhibiting effect on fertilization. (3) I have tested the effect of egg-albumen in the sea-water up to 0.15 per cent without finding any inhibition of fertilization. Of course in higher concentrations a certain degree of inhibition may occur. However the reasons given under 1 and 2 above, certainly exclude the idea that we are dealing with an effect common to colloids in general, which must be assumed to operate through changes in the membrane. It is necessary to assume some specific substance acting as inhibitor.

Fortunately the idea that the inhibitor operates by occupying an ovophile side-chain of the fertilizin is capable of a ready test, for then it should be possible to neutralize the inhibiting effect of the blood, which now becomes ex. hyp. a deviation effect, by treating the inhibiting blood with free fertilizin. This substance should bind all molecules of the inhibitor so that they could no longer exert any deviating effect, their bonds for fertilizin being already occupied. This experiment succeeds perfectly as a matter of fact.

Before citing the experiments in detail, I would like to dwell a little more on their significance. In the first place a negative result would go far to overthrow the entire theory, for then it would become necessary to assume, if the theory were to be retained, that the combination of fertilizin and inhibitor could take place in the egg but not in the test tube, an exceedingly improbable assumption.* The positive result, actually obtained, is in accord with the theory, and it is exceedingly difficult to frame any other interpretation of such a result. The alternative point of view would be to postulate a neutralization of a hypothetical membrane effect of the inhibitor, a conception exceedingly difficult to support as we have seen, and now rendered doubly difficult through the neutralization of such effect by excess of a substance which by itself in high concentrations actually reduces the percentage of fertilizations. Such an alternative interpretation appears to me impossible.

The experiments

July 21, 1913: The following substances were prepared: (1) Fresh filtered blood of male sea-urchins. (2) Filtrate from 15 cc. sea-water plus 7.5 cc. ripe *Arbacia* eggs, i.e., an agglutinating solution in sea-water, which gave an 8 second reaction with fresh sperm at 1/1600 dilution. (3) Filtrate from 15 cc. of the same male blood as in 1, plus 7.5 cc. eggs; i.e., part of the blood (1) saturated with the egg secretions. It gave a 10 second agglutinating test at 1/1600 dilution.

Two drops of fresh sperm (50 per cent) were then stirred into 5 cc. of each of the above and into (4) 5 cc. sea-water for control. These sperm suspensions prepared at 10:50 A.M. were thus about 0.6 per cent. One drop of double washed eggs was then added to each sperm suspension 1-4 at 11:45.

TABLE 10

		SEGMENTED EGGS
(1)	0.6 per cent sperm suspension in blood.....	0.5 per cent
(2)	0.6 per cent sperm suspension in 1600 agglut. power sea-water.....	99.0 + per cent
(3)	0.6 per cent sperm suspension in 1600 agglut. power blood.....	100 0 per cent
(4)	0.6 per cent sperm suspension in sea-water (control)...	100.0 per cent

This experiment was unusually successful; the eggs and sperm were perfect, giving 100 per cent segmentations (4). The blood contained so much inhibitor that only 0.5 per cent of the eggs segmented (1). But its action was entirely neutralized by the addition of the agglutinating substance (3). Fertilization 2 shows that in the presence of such an excess of sperm, the agglutinating substance itself has but little inhibiting power.

In a similar experiment of July 19, 0.9 per cent sperm suspensions were made in solutions 1 to 6 below, and a drop of eggs added to each with results noted in table 11.

TABLE 11

		PERCENTAGE OF SEGMENTED EGGS
(1)	Filtered blood of male sea-urchins	0
(2)	Filtered blood of female sea-urchins	0
(3)	800 power agglutinating solution in sea-water	93%
(4)	1600 power agglutinating solution in mixed blood of males and females.	73%
(5)	Mixture of equal parts of 1 and 3	0
(6)	50 % dilution of 1 with sea-water	0
(7)	Sea-water control	97%

1, 2 and 6 show the inhibiting power of the blood on fertilization even with such enormous excess of sperm; 4 shows neutralization of the inhibiting power by a large quantity of the agglutinating substance although the neutralization was apparently not complete (cf. control); 5 shows that the agglutinating substance contained in an equal part of 800 power sea-water solution was inadequate to neutralize the inhibitor in this sample of blood; a considerable part of the inhibitor must have remained free; it must be present therefore, in high concentration.

Experiments were then made to test the neutralization of the inhibiting power of the blood at various dilutions.

July 22: Used filtered male blood which permitted only 0.5 per cent fertilization. Part of this was saturated with agglutinating substance by addition of 50 per cent by bulk of eggs, and filtered. Fertilizations were then made in a series of sea-water dilutions of each, using a single lot of eggs and of sperm (table 12).

TABLE 12

A SERIES—BLOOD ALONE			B SERIES—BLOOD SATURATED WITH AGGLUTINATING SUBSTANCE ABOUT 6400 STRONG		
Fertilizations in 0.1% sperm suspensions in		Per cent segmented	Fertilizations in 0.1% sperm suspensions in		Per cent segmented
(1)	1% blood	89.0%	(1)	1%	99.0%
(2)	3%	21.0%	(2)	3%	89.5%
(3)	5%	0	(3)	5%	97.0%
(4)	7%	6.0%	(4)	7%	94.5%
(5)	9%	19.0%	(5)	9%	93.5%
(6)	11%	0	(6)	11%	94.0%
(7)	13%	0.1%	(7)	13%	87.5%
(8)	15%	0	(8)	15%	91.0%
(9)	20%	1.75%	(9)	20%	71.0%
(10)	100%	0	(10)	100%	3.0%
Control in sea-water 99%					

It will be seen that this sample of blood is very powerful in inhibitor (A series), and that the agglutinating substance protects from its action at every concentration. The decreases in percentage of segmented eggs in B9 and B10 are to be attributed probably to excess of the agglutinating substance, which was exceedingly concentrated, and which always has some diminishing effect on percentage of fertilizations when the sperm suspension is not too concentrated. In this experiment it will be noted that the agglutinating substance was 6400 strong as contrasted with 1600 on July 21, and the sperm suspension was six times more dilute.

July 25: (A) Used filtered blood of females; (B) part of A saturated with agglutinating substance by addition of eggs; tested 3200 strong. Fertilizations were made in 0.2 per cent sperm suspensions in the following dilutions of each, with per cent of segmented eggs as noted, (table 13):

TABLE 13

A SERIES			B SERIES		
	DILUTIONS	PERCENTAGE OF SEGMENTED EGGS		DILUTIONS	PERCENTAGE OF SEGMENTED EGGS
(1)	10%	17%	(1)	10%	73.5%
(2)	40%	5%	(2)	40%	58%
(3)	70%	0	(3)	70%	40%
(4)	100%	0	(4)	100%	6%
(5)	Control in sea-water 58.5%				

The same protective action of the agglutinating substance is shown, with some decrease at the higher concentrations due, presumably, to excess of the agglutinating substance. The fertilization B-1 actually is better than the control.

August 6: This experiment was made up of a series of combinations of blood A plus fertilizin-saturated blood B. Fertilizations made in 0.2 per cent sperm suspensions in each combination.

TABLE 14

(A) Filtered serum of males and females
 (B) 23 cc. A plus 11.5 cc. ripe eggs. Centrifuged after 10 min. and filtered;
 sperm-agglutination test, $1/3200=9$ sec.

	FERTILIZATIONS IN	PERCENTAGES OF SEG- MENTATION; 400 OF EACH COUNTED, 200 BY MISS BROCKET, 200 BY SELF
	5.0 cc. A + 0 B	
(1)	4.9 cc. A + 0.1 cc. B	0.5%
(2)	4.8 cc. A + 0.2 cc. B	0.5%
(3)	4.7 cc. A + 0.3 cc. B	1.75%
(4)	4.6 cc. A + 0.4 cc. B	8.5%
(5)	4.5 cc. A + 0.5 cc. B	25.0%
(6)	4.4 cc. A + 0.6 cc. B	32.75%
(7)	4.3 cc. A + 0.7 cc. B	31.75%
(8)	4.2 cc. A + 0.8 cc. B	72.0%
(9)	4.1 cc. A + 0.9 cc. B	62.25%
(10)	4.0 cc. A + 1.0 cc. B	35.0%
(11)	3.0 cc. A + 2.0 cc. B	82.75%
(12)	2.0 cc. A + 3.0 cc. B	80.5%
(13)	0 A + 5.0 cc. B	50.0%
(14)		30.25%
	Control in sea-water	89.5%

The protective action of the agglutinating substance gradually rises up to number 10 and then falls off, owing, presumably, to excess of agglutinating substance.

I have given a considerable number of the experiments because the matter under consideration is of great significance. There can be no question that some substance derived from the eggs themselves protects against the inhibiting substance in the blood. Until some method is devised for obtaining the agglutinating substance pure, it is impossible to meet entirely the objection that it may be some substance other than the agglutinating substance that protects against the inhibitor. The agglutinating substance can, however, be demonstrated in high concentrations, as the protocols of the experiments show. Until some reason for a contrary assumption is shown, the neutralization of the inhibitor

must be attributed to this, which is indeed the only assumption consistent with the other known facts.

Now if the agglutinating substance protects from the inhibitor by occupying its combining group, it would be logical to expect to find in some tissue of the species another kind of molecule possessing an identical side-chain and hence equally capable of protecting against the inhibitor. Such a substance was found in the shell but not in the intestine. A sample experiment may be cited.

August 2, 1913: A large quantity of blood was collected from females, and the serum filtered. (A) One part set aside. (B) A second part was saturated with the agglutinating substance by addition of eggs; *B* tested to 1/3200; (C) To a third part was added a large quantity of the shell and peristomes of the sea-urchins used: *B* and *C* filtered again.

Fertilizations were then made in 0.2 per cent sperm suspensions in identical concentrations of each in sea-water as shown in table 15.

TABLE 15

	DILUTIONS OF SERA	PERCENTAGES OF EGGS SEGMENTED		
		A	B	C
(1)	1%	98.0%	98%	97%
(2)	10%	94.0%	99+	96%
(3)	50%	58.5%	96%	99%
(4)	100%	15.0%	94%	97%
Control fertilized in		sea-water 99%		

The conclusion would seem to be that shell secretions (poison from pedicellariae?) protect against the inhibitor in the blood. The inhibitor was not very strong in this case, and test of *C* showed that it contained a small quantity of agglutinating substance owing, as sometimes happened, to injuries to the ovaries in collecting the blood, but there did not seem to be enough (7 second reaction undiluted) to influence the result.

The above result was obtained in an attempt to meet the possible objection that the inhibiting action of the blood might be due to the inclusion of poison from pedicellariae. It appears, on the contrary, that shell-secretions decrease the inhibiting action of the blood, an entirely unexpected result.

As the result met the anticipated objection and did not appear to lie in the main line of the research, the experiment was performed only three times in all, with essentially similar results.

To explain such a result one of two hypotheses is possible: (1) that shell secretions destroy the inhibitor; (2) that they unite with its inhibiting side-chain, and thus possess a combining group identical with the ovophile group of the fertilizin; though they do not possess the spermophile group. The reaction takes place rapidly; hence the second assumption is more likely.

3. SUMMARY OF PART III

In this section we have shown:

1. That the blood of male and female sea-urchins contains a varying amount of a substance which inhibits fertilization without exerting any injurious effect upon either the sperm or the ova. This substance is probably more abundant in the blood of mature individuals than in others.

2. The inhibitor does not act by blocking the combination of the sperm receptors with the fertilizin, for it has no inhibiting effect upon the agglutinating action of the fertilizin.

3. It acts by occupying the ovophile group of the fertilizin, thus preventing action of the latter upon the egg by union with egg receptors.

4. This conclusion is demonstrated by the fact that the inhibitor may be entirely neutralized by a sufficient quantity of the agglutinating substance.

IV. GENERAL DISCUSSION

The essential conception that results from the experiments is that the formation of the fertilization membrane is due to activation of an ovogenous substance, which I have named fertilizin to express this idea. Following insemination any excess of the fertilizin is bound, or neutralized, by another ovogenous substance, which I have named antifertilizin, and polyspermy is thereby prevented. The experiments demonstrate that reactions in fertilization occur as described in sections II and III, but the

intimate nature of the reactions themselves is unknown. To represent them in terms of the Ehrlich hypothesis as definite lock-and-key chemical combinations is of course to go beyond the facts. But it is necessary in some way to gain definiteness of formulation not only for purposes of description, but also to obtain a definite working hypothesis. The method of formulation which I have adopted has both of these advantages. Indeed some of the experiments could hardly have been suggested by any other hypothesis.

1. THE MECHANISM OF FERTILIZATION

In terms of such an hypothesis the mechanism of fertilization may be summed up in a diagram (fig. 1, sector 1). The cortex of the egg contains the fertilizin (see explanation of symbols) of which three molecules are represented each with a spermophile

Fig. 1 In successive sectors of the egg there are represented the mechanism of fertilization and the blocks to the mechanism, as follows:

Sector 1 The arrangement of substances in the unfertilized egg and in the spermatozoon that are active in fertilization. See explanation of symbols.

Sector 2 The mechanism of normal fertilization. The sperm receptor unites with the spermophile group of the fertilizin and the egg-receptors with the ovophile group of the fertilizin owing to activation of the latter by the sperm (a). Molecules of the anti-fertilizin combine with the spermophile group of the adjacent fertilizin (b and c) and thus block the way for supernumerary spermatozoa. This is the postulated mechanism for prevention of polyspermy. At the same time molecules b and c of the fertilizin have also united with the egg-receptors.

Sector 3 Inhibition of fertilization by loss of the active body, fertilizin.

Sector 4 Theory of antagonistic action of spermatozoa of different phyla. The sperm receptors are occupied by combining groups cast off by the antagonistic spermatozoa.

Sector 5 Fertilization is blocked by occupancy of the egg-receptors. Purely hypothetical.

Sector 6 Theory of inhibitory action of blood of the same species. The ovophile group of the fertilizin is occupied by molecules in the blood (inhibitor) possessing the same combining group as the egg-receptors. Molecules of the blood inhibitor also shown in the medium.

The fertilizin is represented in the diagram as occurring only in the cortex of the egg. But it also occurs in high concentration in the jelly surrounding the egg. The spermatozoon must thus normally arrive at the egg-membrane loaded with combined fertilizin. This fact, however, makes no essential difference in the theory, and its representation would complicate the diagram.

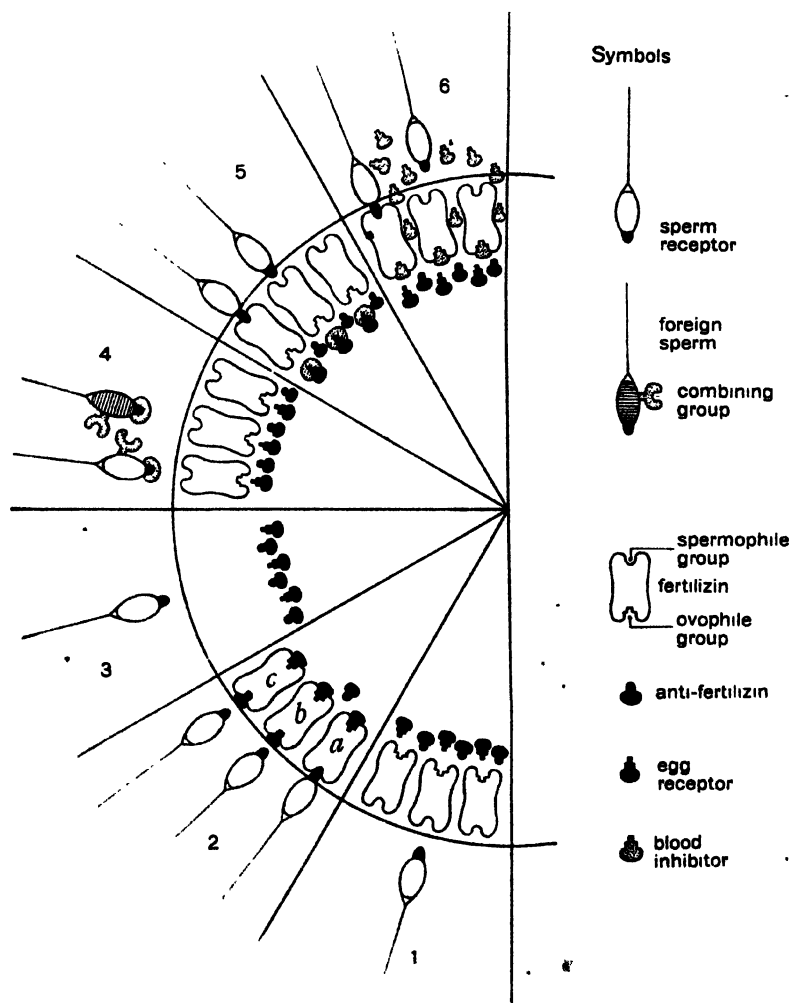


Figure 1

and an ovophile combining group. The spermatozoon is represented with a receptor capable of fitting the spermophile group of the fertilizin; the egg similarly contains receptors capable of fitting the ovophile group of the fertilizin. Within the egg represented as alternating with the egg-receptors, are also found certain molecules (anti-fertilizin) capable of uniting with the spermophile group of the fertilizin.

Union of a sperm receptor with a spermophile group of the fertilizin molecule activates the latter so that the ovophile group forms a union with an egg-receptor (fig. 1, sector 2, *a*); and I have postulated that the fertilizing action is the result of this union. But the activation of the fertilizin can by no means be regarded as confined to those molecules bound by the single penetrating spermatozoon. On the contrary, as we have seen that all the spermatozoa used in insemination bind only a minute proportion of the fertilizin contained in the eggs, it is necessary to assume that the activation of the fertilizin spreads beyond the point of attack of the successful spermatozoon; this is represented in figure 1, sector 2, *b* and *c*. Inasmuch as membrane formation is practically instantaneous all around the cortex, the activation of the fertilizin must spread with extreme rapidity; an activated molecule of the fertilizin must transmit the activated condition to its neighbor, in a fashion that may be compared by analogy to the spread of a stimulus. The assumption here is no different in principle from that which must be made in any theory of the fertilizing effect of the spermatozoon, and therefore, no new difficulty is introduced.

• The postulated mechanism admits of five blocks in the process of fertilization viz.: 1. Absence of the fertilizin (fig. 1, sector 3); 2. Occupancy of the sperm receptors (fig. 1, sector 4); 3. Occupancy of the egg receptors (fig. 1, sector 5); 4. Occupancy of the ovophile side-chain of the fertilizin (fig. 1, sector 6); 5. Occupancy of the spermophile side-chain (fig. 1, sector 2, *b* and *c*). Of these we have examined and discussed in the body of this paper the first, fourth, and fifth, the first in the case of the long washed eggs, (p. 549) the fourth in the case of the inhibitor contained in the blood (p. 563), and the fifth in the case of the non-fertilizable

condition of the already fertilized eggs or those with membranes formed by other methods (p. 556). These cases have been sufficiently discussed already.

But there are two methods of blocking the mechanism according to the hypothesis, which we have not found, viz.: 2 (*supra*) the occupancy of the sperm receptors in such a way that these side-chains are no longer free to unite with the spermophile group of the fertilizin, which would result in loss of fertilizing power of the spermatozoon (fig. 1, sector 4); and 3 (*supra*), viz.: occupancy of the egg receptors, or molecules of the protoplasm with which the fertilizin reacts (fig. 1, sector 5). The discovery of these missing links in the theory would serve as strong confirmatory evidence of its essential correctness.

The latter of these two missing links remains purely hypothetical, but I think it is possible that the former may be represented by the phenomenon of the antagonism of sperm suspensions of different animal phyla as described first by Godlewski ('10 and '11) and subsequently by Herlant ('12, *a* and *b*).

These two authors give quite different interpretations of the results. While Godlewski, relying on the analogy of the antagonistic action of heterogenous hemolytic sera, regards the phenomenon as proof of Loeb's 'lysin theory' of fertilization, Herlant is more conservative, and believes that the explanation cannot be sought in any fundamental alteration of either sexual element; but is rather to be found in a purely 'humoral mechanism,' (i.e., in the effect of substance accompanying the spermatozoa) which he supposes to modify the physical state of the surface of the eggs in such way that the spermatozoa cannot penetrate. Herlant apparently overlooks the fact that penetration of the spermatozoon is not necessary for the cortical changes, as has been shown both by Loeb ('09 and '13) and myself ('12). There is nothing in the results of either author inconsistent with the idea, that the mechanism of inhibition in this case may be due to what I have called, "occupancy of the sperm-receptors" as indicated in sector 4 of the diagram. This may be taken to mean merely such mutual action of the spermatozoa (or extractives thereof) on each other as to inhibit the union with the fertilizin.

This could readily be tested experimentally, for, in such a case, the mixed sperm suspension should no longer agglutinate on exposure to the fertilizin of the eggs used in the experiment. If, for instance, we were to find that sperm suspensions of *Arbacia* and *Nereis* were antagonistic in the sense of Godlewsky, so that mixtures of the two would not fertilize *Arbacia* eggs, then according to my hypothesis the *Arbacia* sperm in the inhibited mixture would not agglutinate with *Arbacia* fertilizin. The experiment yet remains to be performed.

2. THE TWO PHASES OF FERTILIZATION

In the second study of this series (Lillie '11) I showed that fertilization involves two phases in the case of the egg of *Nereis*; the first phase is accomplished before the penetration of the spermatozoon, and leads to changes in the cortex of the egg analogous to membrane formation in the sea-urchins. If the spermatozoon be then prevented from entering, the egg completes the maturation process only and does not segment. The cleavage of the egg is caused by some action of the spermatozoon after penetration. Loeb had also shown by entirely different methods the existence of two similar phases in the fertilization of the ovum of the sea-urchin. *

The question, therefore, arises, does the mechanism of fertilization discussed in this paper deal only with the first, or with both phases of fertilization? To this it must be answered that the mechanism in question is primarily the mechanism of membrane formation. The described results demonstrate this so positively that farther discussion is superfluous on this point.

But the question remains whether there is any relation of such cortical changes to the internal phase of fertilization? Whether, for instance, if the spermatozoon could enter the egg without first forming the combination with the cortical fertilizin, it would then be in condition to carry out the second phase of fertilization, and cause segmentation of the egg? It has been shown, as is well known, that the egg can be induced to develop apparently without the cortical changes involved in membrane formation,

by the use of hypertonic sea-water for instance. It therefore seems possible that the two phases of fertilization may be disconnected in the case of the spermatozoon also, activated perhaps by different parts of it. On the other hand there is the possibility that fertilization is a continuous process and that the second step cannot proceed if the first be skipped; that the spermatozoon in other words receives some necessary modification, by union with the fertilizin or other substances experienced in the cortex of the egg.

The latter point of view seems more probable to me for the following reasons. In the first place I have made some yet unpublished observations in the case of *Nereis* which show that if the cortical changes be induced by artificial means there is a brief period in which insemination of the eggs may be followed by penetration of the spermatozoon, but without causing cleavage of the egg. Such a result might conceivably be due to a lack of synchrony between the movements of the egg-nucleus and the sperm-nucleus owing to the start given the egg-nucleus by the treatment prior to insemination. This explanation does not seem to me to be entirely reasonable, because, in *Nereis*, the sperm enters during maturation and has therefore abundance of time to prepare for union with the egg-nucleus. It seems more probable that the egg has lost something which renders the introduced sperm inefficient. As a matter of fact, all the fertilizin is thrown off or neutralized in *Nereis* during the cortical changes, as I have determined by numerous experiments. Miss Allyn ('12) also determined for *Chaetopterus* that potassium chloride, which starts parthenogenetic development in this form, "initiates changes which increase with time and which are inimical to normal fertilization. They do not prevent the entrance of the sperm into the egg, but they prevent the normal behavior of the sperm in the egg." The cortical changes are not very obvious in *Chaetopterus*, but it is reasonable to suppose by analogy that in initiating parthenogenesis the fertilizin is bound; the sperm entering after this event are relatively inefficient.

Dr. G. L. Kite kindly allows me to refer to some yet unpublished experiments performed last summer in which he injected,

by means of the Barber apparatus, from two or three to over twenty spermatozoa of the star-fish into eggs of the same species. There was never any indication of cleavage. The experiments were limited owing to interruption of Dr. Kite's work, so that the evidence in this case is not yet wholly conclusive; but the results indicate the necessity for normal penetration, if the spermatozoon is to be effective.

We therefore see a second function of the fertilizin, viz.: to prepare the spermatozoon for exercising fertilization effect in the interior of the egg. *The spermatozoon needs itself to be fertilized.*

De Meyer ('11) has observed swelling of the head of the spermatozoon under the action of egg-extractives, and I have described the same thing for *Nercis* ('13). But how such changes intervene in the internal fertilization reaction remains problematical. It is improbable that the swelling itself is significant; it is rather to be taken as indication of a more deeply seated physiological change which is essential for the completion of subsequent reactions.

3. THE LYSIN THEORY OF FERTILIZATION

Loeb's lysin theory of fertilization may be taken as the type of all theories which postulate that the spermatozoon produces and bears a fertilizing substance. Loeb holds that the spermatozoon causes membrane formation and thus initiates development by virtue of a lysin, and he holds this idea not because he, or some other, has isolated this substance and tested its action, but because the action of the spermatozoon in initiating development resembles the action of certain cytolytic substances, more especially, fatty acids, certain glucosides and the blood of some foreign species. Indeed he holds that all cytolytic agents induce membrane formation, and the action of the spermatozoon is regarded as merely a special instance. The spermatozoon, according to Loeb, introduces a second substance into the egg whose function it is to inhibit the harmful effects of membrane formation. However the effect of fertilization in increasing the rate of oxidation within the egg is due not to a catalyzer introduced by the spermatozoon but to the activation of catalyzers

already in the egg as shown by the fact that the rate of development is not increased by superposing fertilization on parthenogenesis or by polyspermic fertilization.

The evidence for the existence of a fertilizing lysin in the sperm is thus entirely analogical and would apply equally well to the conception that the sperm causes membrane formation by activating a substance in the egg, which would make the first part of Loeb's theory of fertilization consistent with the last part. Direct evidence is lacking for the lysin theory, but the whole series of facts described in this paper is direct evidence for the existence of the fertilizing substance in the ovum. We have obtained it in sea-water solution and we have studied its effects both on the spermatozoon and on the ovum and have found it to answer to all the necessary requirements. If the conclusions are accepted, we can hardly continue to believe in the existence of the hypothetical sperm-carried lysin.

But apart from this consideration, the lysin theory exhibits certain features of inadequacy. In the first place the failure of attempts to isolate such a substance from spermatozoa and obtain fertilization can be explained, as Loeb has pointed out, on the assumption that, though presumably present in sperm extracts, it lacks power of penetration in the isolated condition and needs the penetrative power of the living spermatozoon to carry it through the egg membrane. A similar assumption would have to be made with reference to the sperm receptors in my theory; so that this lacuna is not peculiar to the lysin theory.

In the second place the lysin theory affords no explanation whatever of the non-fertilizable condition of fertilized eggs. If membrane formation is the result of a superficial cytolysis produced directly by a lysin carried by the sperm and checked by operation of a second spermatic substance effective after penetration of the spermatozoon, what is to prevent a second cytolysis by a second insemination? There is nothing in the lysin theory that affords the least explanation of this universal phenomenon; even concentrated sperm suspensions do not refertilize or exert a 'cytolytic' effect on fertilized eggs.

Loeb has noted that it is impossible to ascribe the increase of rate of oxidation after fertilization to the introduction of a cata-

lyzer by the sperm because polyspermy does not increase the rate of development over the normal. But he has neglected to note that the same argument applies to his lysin theory; if this theory were correct we should expect that polyspermy would increase the cortical cytolysis above the normal, and result in an unusually distended fertilization membrane; but this is not the case; membrane formation is not excessive, so far as observed, in polyspermic eggs. Yet the lysin theory requires that this should be the case.

It is also inconceivable that a lysin could act effectively in such high dilutions as the respective bulks of spermatozoon and ovum render necessary. Wilson estimates the bulk of the spermatozoon at about 1/400,000 that of the egg in the sea-urchin. The sperm head is not visibly diminished after entrance so it would be unreasonable to suppose that more than 10 per cent of its substance has been used in membrane formation. The lysin theory supposes that a spermatozoon cytolyzes a bulk of 4,000,000 times its lysin content! Not only so, but inasmuch as it is introduced at one point the lysin must diffuse with a speed and evenness that renders it effective simultaneously, and very quickly, at all points on the surface of the egg. This conception seems to me to be inconceivable, for the theory of lysin action presupposes a union molecule for molecule over the affected surface. We should also have a large spermatozoon for large eggs and a small spermatozoon for small eggs on the basis of a lysin theory, which is not the case.

These considerations are arguments not only against the lysin theory but also against any theory that presupposes that the spermatozoon is the bearer of a substance acting directly in causing formation of the cortical changes of fertilization.⁵

I may be allowed finally to point out that, even if some form of sperm extract should be shown to be effective in the production of the cortical change, such a result would be as consistent with the theory of indirect action or activation of an ovogenous substance as with the theory of direct action and would be no argument against the preceding considerations.

⁵ Although Professor Loeb has committed himself very definitely to the lysin theory, he has not failed to note the theoretical possibility of the activation hypothesis (see Loeb 1913, p. 235; earlier reference given here).

4. THE THEORY OF PARTHENOGENESIS

A theory of parthenogenesis must also be a theory of fertilization, at least for the phenomena common to both. Similarly a theory of fertilization must be consistent with the facts of parthenogenesis. This has been generally recognized and it is one of Loeb's great services to have brought about the recognition of this principle and to bring the problem of fertilization definitely into the field of physiology. That the fertilizin theory is consistent in this respect is sufficiently obvious; indeed it makes the action of the sperm itself into a kind of parthenogenesis, for the sperm activates the fertilizing substance already present in the egg. The egg is self-fertilizing.

However, it lies beyond the province of this paper to consider the various methods of artificial parthenogenesis in detail and to examine the question of their consistency with the theory. It is obvious, I think, that the fertilizin theory from its very nature is more elastic with reference to this problem than any other, and is in general, therefore, more consistent with the established fact of the great variety of proved parthenogenetic agents.

Obviously it suggests certain lines of experimentation in parthenogenesis. Glaser has already followed one of these lines with some success, viz.: The effect of exposure of eggs to extracts or secretions of eggs of their own species; and he has ascertained that such extracts are rather efficient parthenogenetic agents in the case of *Arbacia*, especially when followed by treatment with hypertonic sea-water. He did not, however, observe the formation of membranes in eggs so treated, which leaves the mechanism of action rather obscure; farther analysis of the result is desirable.

But the whole procedure in experiments in parthenogenesis must take on a different aspect as soon as it is realized that the entire process, and not merely part of it, consists in acceleration of possible combinations in the egg, or increase in avidity of certain chemical groups.

It is not possible to separate the problem of fertilization from the general problems of cellular physiology; I may therefore be

allowed to point out the impossibility of regarding the interchange between the egg and its environment as controlled exclusively by diffusion through a semi-permeable membrane. However significant the rôle of permeability of the membrane may be, my experiments show that the egg cell is by no means bound in its exchange to diffusible substances; but that the membrane must be regarded as an active factor, and not only a passive one. Twenty years ago diffusion was assumed to play the chief rôle in the absorption from the intestine or in excretion by the kidney epithelium. Today we know that the excretion of urea means work by the kidney cell; it is an active, not a passive, process. Similarly the secretion of fertilizin by the egg cell, and possibly other cortical phenomena, must be regarded as active processes.

The ordinary chemical analysis of the cell begins with destruction of its more highly organized living constituents; it is obvious that such methods are inadequate for the investigation of the immediate reactions in living protoplasm. The results of these experiments may then gain a still broader interest if they may be taken to indicate a method of studying such reactions by the use of living cells as indicators.

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ANTAGONISM BETWEEN SALTS AND ANESTHETICS

IV. INACTIVATION OF SALT SOLUTIONS AND HYPERTONIC SEA-WATER BY ANESTHETICS

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The physiological antagonism about to be described forms a special instance of the class discussed in my three recent papers on the interference of anesthetics with the stimulating and toxic action of salt-solutions.¹ In the second paper I described experiments showing that the cytolytic action of isotonic solutions of various neutral salts on the unfertilized eggs of sea-urchins and starfish was retarded or prevented in the presence of certain anesthetics. The eggs were thus enabled to withstand more prolonged exposures to the anesthetic-containing than to the pure solutions without losing their power of development. Substances of this class may thus exhibit a well-marked protective or anti-cytolytic action similar to that of calcium or magnesium chloride, although as a rule less pronounced.

I have also shown in former papers that the initiation of cleavage by pure isotonic solutions of sodium or potassium salts may be prevented by calcium or magnesium chloride.² This effect is a typical instance of salt-antagonism. There is every indication that the pure or unbalanced salt-solution acts primarily on the plasma-membrane or surface-layer of the egg, causing among other effects a rapid and well-marked increase of permeability, with which is probably associated an electrical depolarization.³ The antagonistic salt counteracts this permeability-increasing action; hence it also prevents the cleavage-initiating

¹ Am. Jour. Physiol., 1912, vol. 29, p. 372; vol. 30, p. 1; 1913, vol. 31, p. 255.

² Am. Jour. Physiol., 1911, vol. 27, p. 289; Jour. Morph., 1911, vol. 22, p. 695.

³ Cf. Am. Jour. Physiol., 1910, vol. 26, p. 106.

action which is dependent upon the change of permeability. Since anesthetics, as well as calcium and magnesium, render the plasma-membrane more resistant to the permeability-increasing action of salt-solutions,⁴ they ought—if the above point of view is correct—also to prevent the cleavage-initiating action of these solutions. It was with this expectation that the following experiments were begun.

A well-marked antagonism of this kind was readily demonstrated in *Arbacia* eggs, although again as with the above anticytolytic action the anesthetics were found to be less effective than calcium or magnesium. The same anesthetics were used as in the preceding experiments with fertilized eggs,⁵ in the concentrations there found just sufficient to prevent cell-division. The addition of anesthetics to the pure salt-solutions used (0.55 m KCNS and NaI) was found in many instances greatly to decrease the cleavage-initiating action of these solutions. Many of the eggs after exposure to the anesthetic-containing salt-solutions for five minutes remained to all appearance unchanged, while those treated similarly with the same salt-solution, free from anesthetic, all formed fertilization-membranes and underwent change of form or cleavage.⁶

The different anesthetics, however, were found to vary greatly in their ability thus to prevent the characteristic action of the salt-solution without injury to the eggs. Chloral hydrate and the urethanes proved much less effective in this respect than the alcohols, although, as shown in my paper on the influence of anesthetics on cleavage,⁷ they are more favorable for anesthetizing the cleavage-process. The ability to counteract the salt-action thus need not run parallel with the power of suppressing cleavage. The case of potassium cyanide affords a further illustration of this difference in the influence of the same compound on these two processes. Cyanide does not appreciably interfere

⁴ Loc. cit., 1912.

⁵ Cf. Jour. Biol. Chem., 1914, vol. 17, p. 121.

⁶ After-treatment with hypertonic sea-water is, of course, necessary to make such eggs develop to advanced stages.

⁷ Loc. cit., 1914.

with the initiation of cleavage by the above salts, in spite of its powerful action in suppressing cleavage—a fact, it may incidentally be pointed out, indicating once more that this agent differs essentially in its mode of action from the anesthetics. That the normal course of the cleavage process is completely inhibited by anesthetics which only slightly counteract the salt-action probably indicates that a greater degree of resistance must be imparted to the plasma-membrane to prevent the action of the salt than to suppress cleavage; chloral hydrate and urethane are apparently less able than the alcohols to impart to the membranes the necessary stability or degree of resistance. The case of cyanide is different. Cyanide acts by directly inhibiting oxidations, and arrests cleavage through a quite different means from that exercised by the anesthetics, which appear to act primarily by altering the condition of the plasma-membrane and thus incapacitating this structure for the exercise of its normal activities.

Precisely how this effect is produced is undecided and requires further investigation; in some way—as I have shown in the experiments cited above—the presence of definite quantities of many lipoid-solvent anesthetics imparts an increased stability to the colloidal surface-films of cells and to other surface-structures such as cilia. Some change in the physico-chemical relations existing between the lipoid and protein components of the colloidal complex seems thus indicated. It has long been known that one colloid may exert a stabilizing influence upon another when the two are present together in solution. The lipoids and proteins of the plasma-membrane are possibly interrelated in some such manner;^a if so, altering the condition of the lipoids will naturally affect the stability of the colloidal system, and under appropriate conditions (of temperature, concentration, etc.) will increase it. When this occurs, activities dependent on alterations of the plasma-membrane—especially the effects normally following changes of electrical polarization—are inhibited. The resulting state of temporary inactivity or irresponsiveness—what we call anesthesia or narcosis in irritable

^a The observations of Lepeschkin are of much interest in this connection; cf. *Kolloid-Zeitschrift*, 1913, Bd. 13, p. 181.

tissues—appears to be the expression of this change in the physico-chemical properties of the plasma-membranes.

It was interesting to find that although anesthetics may thus inhibit the cleavage-initiating action of neutral salts, they have no appreciable influence on the similar action of the lipoid-solvent fatty acids. This observation has an intimate bearing on the general question of the mechanism of anesthetic action, as well as on that of the nature of the process underlying the initiation of cleavage. The fatty acid evidently acts by influencing the condition of the lipoids, and this effect is not prevented by anesthetics; the salt-action, on the other hand, which presumably affects all of the colloids and particularly the proteins of the membrane, is interfered with by these substances. It would thus seem that the salt and the fatty acid may produce the same effect by an action on different constituents of the plasma-membrane. I shall discuss the possible implications of this difference in the relation of anesthetics to these two cleavage-initiating agencies in the concluding section of this paper.

EXPERIMENTAL

Influence of anesthetics on the cleavage-initiating action of neutral salts (KCNS, NaI)

The procedure in these experiments was essentially as follows. The unfertilized Arbacia eggs were exposed for a brief period (four or five minutes) to the pure isotonic solution of the salt used (0.55 m KCNS or NaI), containing the anesthetic in known concentration; they were then returned to sea-water. Eggs treated in this manner with the pure salt-solution, free from anesthetic, form fertilization-membranes and cleave as described in my former paper; and if treated further with hypertonic sea-water a large proportion form blastulae. But of those eggs treated with the salt-solution containing a favorable anesthetic in the appropriate concentration a considerable and sometimes large proportion, varying with the anesthetic and time of exposure, remain to all appearance quite unaffected, showing no

sign of development or other change. If such eggs are fertilized, even after lying in sea-water for many hours (e.g., overnight) they develop into free-swimming larvae, showing that the eggs have not been essentially injured, but that the salt-solution has simply been prevented by the anesthetic from exerting its usual action. The degree of this preventive or antagonistic influence was estimated by comparing the respective proportions of eggs remaining thus unaltered after exposure to the pure and to the anesthetic-containing salt-solutions. In the majority of experiments eggs were also exposed—about fifteen minutes after the treatment with salt-solution—to hypertonic sea-water (100 volumes sea-water plus 16 volumes 2.5 m NaCl) for twenty minutes. The effect of the addition of anesthetics on the action of the hypertonic sea-water was also investigated. In most of the experiments the eggs were anesthetized in sea-water previously to being exposed to the anesthetic-containing salt-solution, i.e., were placed for about half an hour in sea-water containing the same anesthetic in the same concentration as in the salt-solution. The antagonistic influence of the anesthetic is found to be more pronounced after this preliminary anesthetization than when the eggs are transferred directly from normal sea-water to the anesthetic-containing salt-solution.

The details of the manipulation were kept as constant as possible. Equal quantities of eggs from the same lot were used in the different experiments of any series. The anesthetic-containing solutions were prepared shortly before using and kept in corked flasks. The brief exposure to the salt-solution was made in finger-bowls; the pure or anesthetic-containing sea-water was removed as far as possible and to the mass of eggs remaining (usually 2 to 3 cc.) a relatively large volume (usually 50 cc.) of the corresponding salt-solution was rapidly added; at the end of the four or five minutes of exposure a large volume (ca. 300 cc.) of sea-water was added and this was changed as soon as the eggs had settled, and again one or more times later. The sea-water in which the eggs were left after the final treatment was always changed several times to remove the last traces of anesthetic.

Experiments with potassium thiocyanate

Table 1 summarizes the results of two typical series of experiments. These illustrate in a typical manner the characteristic effects of magnesium and calcium in preventing the cleavage-initiating action of the alkali salt; the four alcohols also show a well-marked though less pronounced antagonistic action. The superiority of amyl alcohol and the relative ineffectiveness of ethyl urethane are also typical.

It will be noted that the degree of antagonism is decidedly greater in the case of those eggs (Series B) that were anesthetized previously to the treatment with the salt-solutions. This effect had been foreseen; presumably the preliminary anesthetization alters the plasma-membranes of these eggs, which are thus already in a relatively resistant condition when brought into the salt-solutions; hence the action of the latter on these eggs is more gradual than on eggs transferred to the solutions directly from normal sea-water. In the latter case it is to be assumed that the salt begins to exert its action before the anesthetic has had time to produce its full effect. There is a similar difference in the action of anesthetic-containing sodium chloride solutions on normal and on anesthetized *Arenicola* larvae.⁹ Several other similar experiments gave the same general result.

The further fact appears clearly from these experiments that the anesthetics are less efficient than calcium and magnesium in counteracting the action of the alkali salt. A considerable and variable proportion of the anesthetic-treated eggs form fertilization-membranes and cleave, and later break down like eggs treated with the pure anesthetic-free salt-solution. The proportion of protected and intact eggs may, however, reach 80 or 90 per cent with a favorable anesthetic like amyl alcohol; such eggs appear quite normal and remain without change for many hours; if fertilized they develop into swimming larvae.

The physiological condition of these eggs is, however, not the same as that of normal eggs, but has been altered, apparently in the same general direction as in those eggs which form definite

⁹ Cf. Am. Jour. Physiol., 1912, vol. 29, p. 384.

fertilization-membranes and begin to cleave. This is true also, though to a less degree, of the eggs treated with the calcium- or magnesium-containing solutions. The effects of after-treatment with hypertonic sea-water show the difference between such eggs and normal unfertilized eggs. Hypertonic sea-water acting on normal eggs for this length of time has no apparent effect, causing neither membrane-formation nor cleavage. Its action on eggs previously treated with anesthetic-containing salt-solutions is, however, definite and well-marked. Even those eggs which, if left alone, show no signs of membrane-formation or other change, begin development, and a considerable proportion—though smaller than in the case of eggs with definite fertilization-membranes—reach the blastula stage. Many, of course, die before reaching this stage. Both the above series and that of table 2 afford numerous instances of this kind. It is clear that some physiological modification has been produced in the eggs, of the same nature as that which normally leads to membrane-formation, but insufficient by itself to produce this effect. The eggs are, however, brought into a condition in which they respond more readily to the action of the hypertonic sea-water. The antagonistic effect of the anesthetic is thus only partial; although no membranes are formed and there is no external sign of change, the eggs are rendered more responsive—or sensitized—to the subsequent action of the hypertonic sea-water. Examination of the above and succeeding tables will show that the proportion of eggs remaining unaltered after the treatment with hypertonic sea-water is always small. A certain proportion, however, do remain thus unaltered, apparently those in which the protective action of the anesthetic has been most complete. Such variability is always observed, though its precise basis cannot be defined as yet. Similar conditions are seen in the eggs treated with 0.55 m KCNS containing calcium or magnesium; these cations are decidedly more effective than the anesthetics, as shown above, and of the two magnesium has the greater action; always the great majority (>90 per cent) of eggs treated with these solutions remain to all appearance quite unchanged, and if fertilized the next day develop normally.

TABLE 1

August 13, 1913. In Series A the eggs were transferred directly from normal sea-water into the series of solutions of 0.55 m KCNS containing the anesthetics; they remained in these solutions 4 minutes, and were then returned to sea-water. In addition to the anesthetics, CaCl_2 and MgCl_2 were used for comparison. After the return to sea-water, part of the eggs remained without further treatment; another part were brought, about 15 minutes later, into hypertonic sea-water of the composition 100 volumes sea-water plus 16 volumes 2.5 m NaCl , where they remained for 30 minutes, they were then returned to sea-water. In Series B the treatment was in all respects similar, except that in each experiment the eggs were previously exposed for 30 minutes to sea-water containing the same anesthetic (and additional CaCl_2 and MgCl_2) in the same concentration as in the KCNS solution used in that experiment. From the anesthetic-containing sea-water each lot of eggs was transferred directly to its corresponding KCNS solution. The table describes the condition of the eggs after remaining about 18 hours in sea-water.²

Series A		
SOLUTIONS	CONDITION OF EGGS TREATED WITH SOLUTION ALONE	EGGS TREATED WITH SOLUTIONS FOLLOWED BY HYPERTONIC SEA-WATER
(1) 0.55 m KCNS	Practically all eggs are dead and cytolized	Majority (ca. 60%) form blastulae
(2) 100 vols. 0.55 m KCNS + 10 vols. 0.35 m CaCl_2	Practically all remain unaltered	Ca. 80% remain unaltered; ca. 2-3% form blastulae; the rest cytolized
(3) 100 vols. 0.55 m KCNS + 10 vols. 0.35 m MgCl_2	Practically all remain unaltered	80-90% remain intact; a few form blastulae (< 1%); the rest cytolized
(4) 0.55 m KCNS + 2 vols. % n-propyl alcohol	Most are cytolized: ca. 15-20% unaltered	Ca. 50% form blastulae; very few intact; the rest cytolized
(5) 0.55 m KCNS + 1 vol. % n-butyl alcohol	Most are cytolized; ca. 30-40% intact	Ca. 5% remain intact; ca. 25-30% form blastulae; the rest cytolized
(6) 0.55 m KCNS + 0.4 vol. % i-amyl alcohol ¹	Ca. 30-40% intact; the rest cytolized	A few (ca. 5%) intact; blastulae numerous (ca. 50%); the rest cytolized
(7) 0.55 m KCNS + 0.015% capryl alcohol	Nearly all cytolized; ca. 1% intact	Ca. 30% form blastulae; remainder cytolized
(8) 0.55 m KCNS + 1.25% ethyl urethane	Almost all cytolized; little apparent effect	Large proportion form blastulae (ca. 75%); the rest cytolized

TABLE 1—Continued
Series B

	Same as Series A	Same as Series A
(1) 0.55 m KCNS	Practically no change; all eggs intact	A few blastulae (2-3 %); most eggs intact; some broken down
(2) 100 vols. 0.55 m KCNS + 10 vols. 0.35 m CaCl ₂	Practically all eggs intact	90 % or more remain intact; none form blastulae
(3) 100 vols. 0.55 m KCNS + 10 vols. 0.35 m MgCl ₂	% n-propyl	Few eggs intact; most cytolized; ca. 20 % form blastulae
(4) 0.55 m KCNS + 2 vols. % n-butyl	Ca. 35-40 % intact. The rest cytolized	Like 4B; a few intact; ca. 20 % blastulae
(5) 0.55 m KCNS + 1 vol. % i-amyl	Most eggs remain intact; (ca. 80-90 %)	A small proportion intact (ca. 5 %); ca. 20 % form blastulae; the rest cytolized
(6) 0.55 m KCNS + 0.4 vol. % capryl	Decided difference from 7A; ca. 30-40 % intact	Few intact (< 1 %); ca. 25 % form blastulae; the rest cytolized
(7) 0.55 m KCNS + 0.015 % ethylurethane	A small proportion of intact eggs (ca. 5 %); the rest cytolized	Ca. 50 % form blastulae; the rest cytolized

¹ The amyl alcohol used was iso-amyl (fermentation-amyl) alcohol, (CH₃)₂CH.CH₂.CH₂OH. In my former paper in this journal (1913, vol. 15, p. 23) this compound was inadvertently described as n-amyl alcohol.

² As a further test of the protective action of the anesthetics, spermatozoa were added the next morning (19 hours after the exposure to salt-solution) to the eggs that had been treated with the salt-solutions alone (without hypertonic sea-water). A large proportion of the anesthetic-treated eggs formed blastulae; the number of eggs thus developing was higher in Series B than in Series A and showed a general correspondence with the results indicated in the first column of the table. Most of the eggs from Solutions 2 and 3 developed, and very few from Solution 8; the eggs from the other solutions showed intermediate conditions. A very small proportion of eggs treated with pure 0.55 m KCNS also survived and formed blastulae. The preliminary exposure to anesthetics in sea-water in Series B has no effect on the eggs; all remained unaltered next day and developed normally after fertilization.

Nevertheless, the after-treatment with hypertonic sea-water initiates development in a certain small proportion of these eggs, some of which form blastulae, though most stop short of this stage and undergo cytolysis. It is clear from the altered character of the response to hypertonic sea-water that some persistent modification has been produced by the previous treatment with salt-solution. What the nature of this modification is can only be surmised at present. The most probable general hypothesis seems to be that the plasma-membrane has been altered in some definite way—possibly rendered more permeable or more susceptible to alterations of permeability under changed external conditions. A changed state of electrical polarization would presumably accompany such a modified condition. On this view the increased responsiveness to hypertonic sea-water is analogous to the increased responsiveness of frogs' voluntary muscle which has been sensitized by brief exposure to isotonic solutions of (e.g.) sodium citrate or other sodium salt.¹⁰ This form of sensitization is almost undoubtedly dependent on a surface-alteration, since it is produced within a few seconds by salts which either do not penetrate the normal plasma-membrane or do so with extreme slowness.¹¹

The degree of protection afforded by the above alcohols may be decidedly greater than that shown in the above series. The following experiments (table 2) are especially favorable in this respect. These experiments also bring out clearly the effectiveness of anesthetics in inhibiting the characteristic action of hypertonic sea-water. Ethyl alcohol and phenyl urethane were used in addition to the anesthetics of table 1.

It will be seen from an examination of table 2 that the protective action of the alcohols is well-marked, while that of the urethanes, especially phenyl urethane, is comparatively slight. Amyl alcohol is distinctly superior to the others. These results are typical, as an examination of table 5 will show. A small proportion of the protected eggs remain unaffected by the after-

¹⁰ Cf. J. Loeb, *Am. Jour. Physiol.*, 1901, vol. 5, p. 362.

¹¹ Cf. my paper in the *Proceedings of the Society for Experimental Biology and Medicine*, 1910, vol. 7, p. 170.

TABLE 3

August 16, 1913. Unfertilized *Arbacia* eggs were exposed for about 28 minutes to sea-water containing the following anesthetics in the same concentrations as in the respective salt-solutions. From this sea-water each lot of eggs was transferred directly to its corresponding salt-solution:—0.55 m KCNS containing the anesthetic in the concentration given below. The exposure to the salt-solution was 4 minutes. About 15 minutes later part of the eggs were placed in hypertonic sea-water (100 volumes sea-water plus 16 volumes 2.5 m NaCl), another part (B) in hypertonic sea-water containing the same anesthetic in the same concentration as in the corresponding salt-solution. After 20 minutes in hypertonic sea-water the eggs were returned to normal sea-water. The condition of the eggs on the day following (after about 18 hours) is given in the table.

SOLUTIONS	CONDITION OF EGGS TREATED WITH SOLUTIONS ALONE		CONDITION OF EGGS TREATED WITH HYPERTONIC SEA-WATER	
	(A) free from anesthetic		(B) containing anesthetic	
(1) 0.55 m KCNS	Practically all eggs are dead and cytolyzed		Ca. 50-60 % form blastulae; the rest are broken down	
(2) 0.55 m KCNS + 4 vols. % ethyl alcohol	Ca. 20-30 % remain intact; the rest cytolyzed		Ca. 50 % form blastulae; the rest are broken down	
(3) 0.55 m KCNS + 2 vols. % propyl alcohol	Ca. 25-30 % intact; the rest cytolyzed		Ca. 30 % form blastulae; ca. 5 % remain intact; the rest broken down	
(4) 0.55 m KCNS + 1 vol. % butyl alcohol	Most eggs remain intact (ca. 60-70 %); the rest cytolyzed		Ca. 30 % form blastulae; a few intact (ca. 1 %); the rest broken down	
(5) 0.55 m KCNS + 0.4 vol. % amyl alcohol	Large majority (ca. 80-90 %) intact; the rest cytolyzed		Comparatively few blastulae (ca. 5-10 %). Most eggs cytolyzed. Ca. 5-10 % remain intact	
(6) 0.55 m KCNS + 0.015 % capryl alcohol	Ca. 50-60 % remain intact; the rest cytolyzed		Ca. 20-30 % blastulae; ca. 5 % remain intact; the rest broken down	
(7) 0.55 m KCNS + 1.5 % ethyl urethane	Protection relatively slight; ca. 10 % intact eggs		Large proportion of eggs form blastulae (> 50 %). The rest broken down	
(8) 0.55 m KCNS + 0.06 % phenyl urethane	Little protective action; ca. 1-2 % intact eggs; the rest cytolyzed		Blastulae numerous; ca. 60 % or more; the rest broken down	

Most eggs are dead without signs of development; few (ca. 1 %) form blastulae. Ca. 4-5 % remain intact

Most dead; a few intact (ca. 5-10 %); ca. 2 % form blastulae

Practically all are dead; no blastulae. Ca. 1 % intact

Many remain intact; ca. 30 %. Rest cytolyzed. No blastulae

Most eggs cytolyzed; a few remain intact (2-3 %)

Nearly all dead without development; no blastulae; ca. 1-2 % intact

Nearly all are dead. A few form blastulae, ca. 1-2 %; no intact eggs

treatment with hypertonic sea-water and remain intact the next morning; but the majority either undergo cytolysis or develop to a blastula stage. The proportion of eggs forming blastulae is in general smaller the greater the protective action, indicating that when the membrane-forming action of the salt is prevented the eggs respond less readily to the hypertonic sea-water; some entirely fail to respond.

It will be noted also that the presence of the anesthetic in the hypertonic sea-water greatly diminishes or entirely annuls the effectiveness of the after-treatment. Such hypertonic sea-water is not, however, entirely indifferent in its action, as is shown by the fact that relatively few eggs so treated remain intact; most undergo cytolysis and a few may develop. The same result appeared in six other series of experiments with hypertonic sea-water containing anesthetics. The favorable effect of the treatment is, however, almost entirely prevented by the presence of anesthetics in the protective or anesthetizing concentrations.

Removal of oxygen from hypertonic sea-water or the addition of cyanide also prevents its characteristic action, as Loeb found for *Strongylocentrotus*.¹² Cyanide has the same effect with *Arbacia* eggs (cf. table 3). Loeb has interpreted these facts as indicating that some chemical process involving oxidations underlies the favorable action of the hypertonic sea-water. That this agent acts by modifying chemical processes in the egg is also indicated by the high temperature-coefficient of the times of exposure.¹³ The fact that anesthetics have the same effect on the action of hypertonic sea-water as suppression of oxidations seems highly significant. Evidently the hypertonic sea-water induces some process of an oxidative nature; this process is checked or prevented by anesthetics. Now the anesthetics appear to act by altering the state of the lipid components of the plasma-membrane, thus rendering this structure more resistant to change than normally: it thus appears probable that the

¹² J. Loeb, *Biochemische Zeitschrift*, 1906, Bd. 1, p. 183.

¹³ J. Loeb, loc. cit.; also University of California Publications, *Physiology*, 1906, vol. 3, p. 39.

hypertonic sea-water exerts its characteristic action primarily by changing the state of the plasma-membrane, and that the oxidative processes underlying the favorable effect of this after-treatment are a function of certain membrane-processes. We have thus further though somewhat indirect evidence that the plasma-membrane is a controlling factor in the intracellular oxidations.¹⁴

Chloral hydrate has little effect in preventing the action of 0.55 m KCNS. In five experiments with this anesthetic, in concentrations of 0.2 per cent to 0.1 per cent, the highest proportion of eggs remaining unaltered next day was *ca.* 10 per cent (table 5). Potassium cyanide showed no signs of protective action in any experiment. The series shown in table 3 illustrates the results obtained with these compounds.

It will be noted that although chloral hydrate and cyanide are almost without influence on the cleavage-initiating action of 0.55 m KCNS, both prevent entirely the favorable effects of after-treatment with hypertonic sea-water. This action was highly striking in the above series, since in every experiment the great majority of eggs formed blastulae after treatment with the pure hypertonic sea-water.

Experiments with sodium iodide

In experiments with this salt all the above alcohols showed well-marked protective action. The series summarized in table 4 will illustrate. The eggs were exposed to the freshly prepared 0.55 m NaI for four minutes; this is too brief an exposure to cause membrane-formation in all eggs, and about 20 per cent remained intact next morning; the proportion remaining intact was, however, much greater in the anesthetized lot, and fewer of these eggs formed blastulae.

A second similar series with five minutes' exposure to 0.55 m NaI, and containing in addition to the above alcohols ethyl urethane and chloral hydrate, gave similar results, although the protective effect was on the whole less pronounced. Urethane

¹⁴ For more direct evidence of a relation of membranes to oxidations, cf. my recent paper in the *Journal of Biological Chemistry*, 1913, vol. 15, p. 237.

TABLE 3

August 28, 1918. The procedure was the same as in the series of table 2. The eggs were left in sea-water containing chloral hydrate and cyanide for 30 minutes before transfer to the salt-solutions. Exposure to salt-solutions was 4 minutes; to hypertonic sea-water, 20 minutes.

SOLUTIONS	CONDITION OF EGGS TREATED WITH SOLUTION ALONE	CONDITION OF EGGS TREATED WITH HYPERTONIC SEA-WATER	
		(A) free from anesthetic	(B) containing anesthetic
(1) 0.55 m KCNS	Practically all eggs cytolized	Ca. 75 % form blastulae	None form blastulae; practically all cytolized
(2) 0.55 m KCNS + n/1000 KCN	Practically all eggs cytolized	Ca. 75-85 % form blastulae	None form blastulae; nearly all cytolized; a few intact
(3) 0.55 m KCNS + 0.2 % chloral hydrate	Almost all cytolized; a few intact eggs	Ca. 70-80 % form blastulae	None form blastulae; a few intact
(4) 0.55 m KCNS + 0.15 % chloral hydrate	Slight protective effect; nearly all cytolized	Majority form blastulae (> 75 %)	None form blastulae; a few intact
(5) 0.55 m KCNS + 0.01 % chloral hydrate	Little or no protective effect; practically all eggs cytolized	Same as above; ca. 75-80 % form blastulae	None form blastulae; a few intact

TABLE 4

August 23, 1919. The eggs were exposed to sea-water containing the following anesthetics for 30 minutes before transfer to the salt-solutions. The exposure to the salt-solutions was 4 minutes. After ca. 15 minutes in sea-water the eggs were transferred to hypertonic sea-water, with and without anesthetics, where they remained 20 minutes.

SOLUTIONS	CONDITION OF EGGS TREATED WITH SOLUTIONS ALONE	CONDITION OF EGGS TREATED WITH HYPERTONIC SEA-WATER	
		(A) free from anesthetic	(B) containing anesthetic
(1) 0.55 m NaI	Most eggs are cytolyzed next day; ca. 20 % remain intact	Ca. 30 % form blastulae; ca. 10-15 % remain intact	
(2) 0.55 m NaI + 4 vol % ethyl alcohol	Most eggs are intact next day (ca. 70 %)	Blastulae are fewer than in Exp. 1 (ca. 10-15 %); ca. 20 % remain intact	Large proportion remain intact, > 50 %; a few blastulae < 1 % the rest cytolyzed
(3) 0.55 m NaI + 2 vol. % propyl alcohol	Most eggs remain intact (ca. 60-70 %)	Blastulae few (ca. 5 %); most eggs cytolyzed; small proportion intact	Most eggs cytolyzed; 10-15% intact; no blastulae
(4) 0.55 m NaI + 1 vol % butyl alcohol	Ca. 80 % remain intact	Ca. 10 % form blastulae; ca. 20 % remain intact; the rest cytolyzed	None form blastulae; few (< 5 %) intact; the rest cytolyzed
(5) 0.55 m NaI + 0.4 vol % i-amyl alcohol	Ca. 70-80 % remain intact	Few blastulae (ca. 5 %); ca. 5 % remain intact	None form blastulae; ca. 5-10 % remain intact; remainder cytolyzed
(6) 0.55 m NaI + 0.015 % capryl alcohol	Ca. 50-60 % remain intact	Few blastulae (ca. 5 %); ca. 20 % remain intact	None form blastulae; few intact (ca. 2-3 %); the rest cytolyzed

was only slightly effective, while chloral hydrate showed well-marked action. In all cases the anesthetic prevented or greatly lessened the favorable effects of the after-treatment with hypertonic sea-water. The results with sodium iodide were thus in all respects similar to those with potassium thiocyanate.

In table 5 I have summarized the results of last summer's series of experiments with 0.55 m KCNS. The table includes the records of all experiments in which the eggs were exposed, previously to treatment with the anesthetic-containing salt-solutions to solutions of the same anesthetics in sea-water, in the manner already described.¹⁵ The figures give the estimated proportion of eggs remaining intact the next morning after the treatment with the anesthetic-containing salt-solution alone. In all cases the proportion of eggs surviving the treatment with the pure 0.55 m KCNS (without anesthetic) was small—never more than 1 or 2 per cent.

The concentrations of anesthetic used in these experiments are those which just suffice to prevent cleavage in most fertilized eggs, without causing immediate injury.¹⁶ The above solutions (with the exception of 2.4 v. per cent propyl alcohol and 1.2 v. per cent butyl alcohol, which are too strong) are thus, as regards their inhibiting effect on cleavage approximately equivalent. Nevertheless in their ability to prevent the cleavage-initiating action of salt solutions they show marked inequalities. The alcohols are greatly superior to chloral hydrate and the urethanes; amylalcohol is distinctly more effective than the others, while butyl alcohol appears somewhat more favorable than propyl or capryl, and ethyl is only moderately effective. This lack of parallelism between the anesthetic and the above protective actions may seem inconsistent with the general hypothesis advocated in this paper. But when the essential dissimilarity between the process of normal cleavage and the external action of a pure salt-solution is considered, the discrepancy ceases to be surprising. That

¹⁵ With the exception of a few experiments in which the results of fertilization showed that the eggs were defective.

¹⁶ Cf. my paper on the influence of anesthetics on cleavage cited above, pp. 128 *seq.*

TABLE 5

In all cases the eggs were treated, previously to placing in 0.55 m KCN, with sea-water containing the anesthetic (the same anesthetic in the same concentration as in the salt-solution used in that experiment) for about 30 minutes. The exposure to 0.55 m KCN was 4 minutes. The figures give the estimated proportion of eggs that remained intact after leaving in sea-water over night (i.e., 18-20 hours after exposure to the solution). In all cases the treatment with the pure anesthetic-free 0.55 m KCN left few if any eggs intact next day (always less than 2 %). Each vertical column gives the results of a separate series.

ANESTHETIC SOLUTION IN 0.55 m KCN	NUMBER AND DATE OF SERIES								
	1 August 4	2 August 6	3 August 11	4 August 13	5 August 14	6 August 16	7 August 19	8 August 21	9 August 28
0.2 % chloral hydrate.	<1%								ca. 1%
0.15 % chloral hydrate.									ca. 1%
0.1 % chloral hydrate.	<1%								ca. 1%
4 v. % ethyl alcohol.						20-30%	10-20%	10-15%	
2.4 v. % n-propyl alcohol.		0							
2 v. % n-propyl alcohol.			10-15%	20-25%	5-10%	25-30%	40-50%	10-15%	
1.2 v. % n-butyl alcohol.	ca. 1%	0							
1 v. % n-butyl alcohol.	20-30%		25-30%	35-45%	ca. 10%	60-70%	50-60%	20-30%	
0.4 v. % i-amyl alcohol.	50%	>50%	50%	80-90%	ca. 80%	80-90%	40-50%	ca. 10%	
0.015 % capryl alcohol.		40-50%	10%	30-40%	20-30%	50-60%	ca. 30%	ca. 10%	
0.2 % chlorotone.		0							
1.75 % ethyl urethane.					ca. 5%				
1.5 % ethyl urethane.		20%	ca. 5%	ca. 5%		ca. 10%	5-10%	ca. 10%	
1.25 % ethyl urethane.									
0.06 % phenyl urethane.					ca. 1%	1-2 %	2-3 %	ca. 5%	

a given anesthetic may interfere with one set of processes and not with another has long been known. It would appear rather, as suggested above, that while chloral hydrate and the urethanes impart to the plasma-membranes a degree of resistance sufficient to prevent the normal changes of cleavage, this resistance is insufficient to prevent the relatively violent action of the pure salt-solution. The alcohols, for some reason as yet obscure, protect the egg more effectively against the latter action. They are, however, quite ineffective in preventing the cleavage-initiating and cytolytic action of fatty acids. The ability of an anesthetic to suppress or prevent a given process thus depends both on the nature of the anesthetic and of the process itself. The experiments with fatty acid about to be described will illustrate this.

The cleavage-initiating action of fatty acids in the presence of anesthetics

The above anesthetics entirely fail to interfere with the formation of fertilization-membranes or the initiation of cleavage by fatty acids. In a number of experiments, conducted similarly to those already described, in which the parthenogenetic agent was sea-water containing acetic or butyric acid (2 to 3 cc. $\frac{1}{2}$ fatty acid plus 50 cc. sea-water) the results were entirely negative. The addition of the anesthetic seems indeed to increase the injurious action of the acid. Chloral hydrate is equally ineffective. The urethanes were not tried. The following series with the alcohols will illustrate (Table 6).

From these experiments it is clear that the above anesthetics are completely unable to counteract the action of the fatty acid. Their antagonistic influence seems to be confined to the salts. Experiments with sea-water containing additional calcium and magnesium chloride (without altering the osmotic pressure) gave an analogous result. It was thought that possibly by increasing the proportion of these salts in the medium the eggs might be rendered more resistant to fatty acid. The following solutions were used: 100 volumes sea-water *plus* respectively.

TABLE 6

August 30, 1913. The eggs were exposed to the solutions of the following alcohols in sea-water for 26 minutes. They were then treated for 3 minutes with sea-water containing $\frac{1}{100}$ butyric acid (50 cc. sea-water plus 2 cc. $\frac{1}{100}$ butyric acid). After an interval of 15 minutes part were exposed to hypertonic sea-water, with and without anesthetics, for 20 minutes. The condition of the eggs after 19 hours was as follows:

SOLUTIONS	CONDITION OF EGGS TREATED WITH SOLUTIONS ALONE	CONDITION OF EGGS AFTER-TREATED WITH HYPERTONIC SEA-WATER	
		(A) without anesthetic	(B) containing anesthetic
(1) Sea-water + $\frac{1}{100}$ butyric acid	All cytolyzed	80-90 % form blastulae	Nearly all cytolyzed; a few blastulae; < 1% time
(2) Same + 4 vol. % ethyl alcohol	All cytolyzed	Blastulae fewer than in Exper. 1; ca. 60-70 % blastulae	All cytolyzed
(3) Same + 2 vol. % n-propyl alcohol	All cytolyzed	Most eggs cytolyzed; ca. 5 % form blastulae	All cytolyzed
(4) Same + 1 v. % n-butyl alcohol	All cytolyzed	None form blastulae; all cytolyzed	All cytolyzed
(5) Same + 0.4 v. % i-amyl alcohol	All cytolyzed	None develop; all cytolyzed	All cytolyzed
(6) Same + 0.015 % capryl alcohol	All cytolyzed	Many form blastulae; ca. 50 %; the rest cytolyzed	Nearly all cytolyzed; one or two blastulae seen

10, 20 and 50 cc. 0.35 m MgCl_2 , similar mixtures of 0.35 m CaCl_2 and sea-water, and mixtures containing both salts; 2 cc. $\frac{N}{10}$ butyric acid was added to 50 cc. of each solution, and the action on unfertilized eggs was tested as above. The results again were entirely negative as regards protective action: in all cases two minutes' exposure to these solutions was followed by cytolysis. After-treatment with hypertonic sea-water proved ineffective in all cases, none of the eggs so treated forming blastulae (with the exception of a few from 100 volumes sea-water plus 10 volumes 0.35 m MgCl_2). The presence of an excess of calcium or magnesium in the sea-water thus prevents the eggs from developing favorably later, although it does not hinder the cytolytic action of the fatty acid.¹⁷

¹⁷ Experiments, with unfertilized eggs conducted in the summer of 1911 on the antitoxic action of CaCl_2 on isotonic NaCl solutions containing acetic acid gave entirely negative results. The following series will illustrate. Eggs were left for two hours in the solutions, then returned to normal sea-water and fertilized. The results were as follows (condition of the eggs next day):

	Solution	Result
(1)	Pure 0.55 m NaCl	ca. 40-50 % form blastulae
(2)	95 vols. 0.55 m NaCl +5 vols. $\frac{M}{2}$ CaCl_2	all form blastulae
(3)	0.55 m NaCl + $\frac{N}{200}$ CH_3COOH	all eggs dead
(4)	0.55 m NaCl + $\frac{N}{200}$ CH_3COOH + $\frac{M}{40}$ CaCl_2	all dead
(5)	0.55 m NaCl + $\frac{N}{400}$ CH_3COOH	all dead
(6)	0.55 m NaCl + $\frac{N}{400}$ CH_3COOH + $\frac{M}{40}$ CaCl_2	all dead
(7)	0.55 m NaCl + $\frac{N}{600}$ CH_3COOH	all dead
(8)	0.55 m NaCl + $\frac{N}{600}$ CH_3COOH + $\frac{M}{40}$ CaCl_2	all dead
(9)	0.55 m NaCl + $\frac{N}{800}$ CH_3COOH	all dead
(10)	0.55 m NaCl + $\frac{N}{800}$ CH_3COOH + $\frac{M}{40}$ CaCl_2	all dead

Thus CaCl_2 in concentrations which completely prevent the toxic action of the NaCl solution has no effect on the toxic action of acetic acid. CaCl_2 also showed no antitoxic action in 0.55m NaCl containing NH_4OH in concentrations from $\frac{N}{40}$ to $\frac{N}{2580}$. It also failed to antagonize HCl in concentrations from $\frac{N}{100}$ to $\frac{N}{2580}$; but with weaker solutions ($\frac{N}{400}$ to $\frac{N}{2580}$ HCl) some antitoxic effect was seen. *Arbacia* eggs thus differ from *Fundulus* eggs, which are protected by salts to a considerable degree against injury by acetic acid in $\frac{N}{33}$ concentration (cf. J. Loeb, *Biochemische Zeitschrift*, 1912, vol. 47, p. 151). *Fundulus* eggs are, however, surrounded by a resistant chorionic membrane.

CONCLUSIONS

We reach thus the general result that the formation of fertilization-membranes and the initiation of cleavage may be prevented by anesthetics when the parthenogenetic agent is a neutral salt, but not when it is a fatty acid. This contrast is what would be expected on the assumption that the essential action of the anesthetic is superficial, and consists in rendering the plasma-membrane more resistant to alterations of permeability. Hence the salt, which does not readily penetrate the unaltered egg and produces its effect by increasing the permeability of the plasma-membrane, is rendered less effective when the membrane has been rendered relatively resistant or stabilized by the anesthetic. The fatty acid, on the other hand, which penetrates the plasma-membrane readily under all conditions, by virtue of its lipoid-solubility, is not prevented in its action by anesthetics.

Fatty acids and neutral salts represent two classes of agents, one of which penetrates the egg-surface readily, the other with difficulty and apparently only after increasing the permeability of the plasma-membrane. Both induce parthenogenesis in a typical manner. It is significant that of these two parthenogenetic agents one should be influenced in its action by anesthetics, the other not. The fact that it is the more penetrating of the two which is uninfluenced seems to indicate that the agent produces its essential effect by acting on some portion of the egg-cytoplasm situated *within* the most external surface-layer, and that calcium and anesthetics inhibit the action of salt-solutions because they prevent the access of the salt to this critical region of the egg. Otherwise it is difficult to understand why the anesthetic, which apparently stabilizes the surface-layer and hinders alteration of permeability, is without influence on the action of the fatty acid, although it inhibits the action of the salt. The entrance of the salt but not of the lipoid-soluble fatty acid would be hindered by agents which act (like anesthetics and Ca salts) by preserving semi-permeability unaltered, since semi-permeability relates to lipoid-insoluble substances¹⁸ only, as Overton first showed.

¹⁸ I.e., non-colloidal substances. Ruhland has shown that various dyes which form colloidal solutions are exceptions to Overton's general rule. Cf. Jahrb. wiss. Botanik, 1908, Bd. 46, p. 1.

It is of course also possible that the actual entrance of the salt into the egg is unnecessary, and that a purely superficial action sufficient to increase permeability to a critical degree and thus cause a definite depolarization-effect is all that is necessary. There is at present no certain means of deciding between these two alternatives. If the salt increases permeability to a sufficient degree, it will naturally enter the egg and produce certain effects in its interior. It is, however, clear that there is nothing specific about the salt-action; all that is needed is that it should be sufficiently energetic. The entrance of special substances from outside into the egg is not necessary for parthenogenesis: the effects of temporary warming and mechanical agitation upon starfish eggs are a sufficient proof of this. On the other hand, certain substances as fatty acids and lipoid-soluble alkalis, do undoubtedly produce their effects by penetrating the egg.¹⁹ The comparative ineffectiveness of the lipoid-insoluble and non-penetrating alkalis and acids indicates this clearly. The most probable conclusion seems to be that the same effect can be produced by a purely superficial action, like that of a salt or the electric current, as by one operating at some region within the interior of the egg.

There are many indications that the primary effect in the activation of the egg—whether by the spermatozoön or a parthenogenetic agent—is superficial and consists in an alteration of the surface-layer of protoplasm—the region somewhat vaguely designated as plasma-membrane. The term ‘plasma-membrane’ in its application to the surface-film seems at present to require more precise definition. The conception of this structure as a thin homogeneous haptogen membrane exercising passive mechanical and osmotic functions is clearly inadequate. It must rather be regarded as essentially a superficial portion of the living protoplasm, characteristically modified in its composition and physical properties by surface forces. We must thus ascribe to it a characteristic chemical organization and metabolism as well as the characteristic physical and other properties, such as

¹⁹ Cf. Loeb's recent paper on "The comparative efficiency of weak and strong bases in artificial parthenogenesis," Jour. Exp. Zool., 1912, vol. 13, p. 577.

selective semi-permeability, that have led to its distinction from the more internal protoplasm. Conceived in this way, it corresponds closely to what morphologists designate as the cortical region of the egg, or at least to the most external layer of this region.

Changes in this region form a highly characteristic accompaniment of fertilization in many if not in all eggs; associated with these changes is a marked temporary increase in the general permeability of the surface-layer. The relation of these surface-changes or cortical processes to the initiation of cell division and development is evidently a critical one.²⁰ Once they are accomplished the developmental mechanism, hitherto held in check, resumes operation and—provided external and other conditions are favorable—continues its course automatically to the adult stage. It is clear, from the diversity of the conditions that may initiate development, that some process specific to the egg and quite independent of the nature of the activating condition forms the primary event in fertilization. The spermatozoön or the parthenogenetic agent in some way removes the hindrance to this process. What the nature of the latter is may be partly inferred from the results of recent experiments on the physiology of fertilization. The observations described in this paper support the view that some change in the cortical region of the egg-protoplasm, beneath the most external semi-permeable layer of plasma-membrane proper, forms the initial stage of the fertilization-process. The immediate surface of the egg has semi-permeable properties relatively to most water-soluble lipoid-insoluble substances, and apparently must undergo increase of permeability in order that such a salt as NaI or KCNS may produce its characteristic effect. As already said, it is uncertain whether the salt acts by entering and then affecting directly the state of the colloids in the cortical region, or whether it acts without entrance, possibly by altering the electrical polarization of the plasma-membrane. It seems probable, however, from the general effectiveness of lipoid-alterants, that some

²⁰ Cf. F. R. Lillie, on The cortical changes in the egg of Nereis. Jour. Morph., 1911, vol. 22, p. 361.

change in the condition of the lipoids—possibly in the inter-relations between lipoids and proteins—is the primary effect produced and that this change then initiates some specific chemical reaction which determines directly or indirectly the characteristic surface-changes of fertilization, namely, secretion of cortical material, formation of fertilization-membrane, temporary change in osmotic properties of the plasma-membrane with accompanying electrical depolarization.

This view emphasizes the analogy between the activation of the resting egg and the general process of stimulation.²¹ In stimulation the primary event is a depolarization of the limiting membrane; similarly in the fertilization-process the electrical variation accompanying the above surface changes forms most probably the critical or 'releasing' event on which the rest of the process automatically follows. One gains the impression that in the resting egg-cell, as well as in the resting muscle or nerve, certain substances are hindered from interacting by the electrical polarization at the cell-surface; just as in a battery with open circuit the chemical reactions on which its operation depends are held in check by the polarization at the surface of the plates;²² that is, the passage of ions into or out of solution is thus prevented and with it all effects, chemical and other, dependent on the flow of electricity through the circuit. Under analogous conditions in the living cell a brief depolarization might suffice to release the impediment to the chemical interaction forming the primary event in the response—whether to stimulation or (in the case of the egg-cell) to fertilization. It is noteworthy that in many if not in most irritable cells the response is specific and constant and independent of the character and intensity of the stimulus; that is, recent research indicates that the "all or none" law applies to irritable elements in general,²³ and not only to heart-muscle, and it may be said

²¹ For a fuller discussion of this analogy, cf. my recent paper in the *Journal of Exp. Zool.*, 1913, vol. 15, p. 23.

²² The solution-tension of the ions being compensated by the electrostatic attraction between the plate and the oppositely charged adjoining layer of solution.

²³ Cf. especially the recent articles from the Cambridge Physiological Laboratory by Lucas and Adrian in the *Journal of Physiology*.

with some qualification to apply also to the resting egg-cell. The removal of an inhibiting condition, whatever the means employed, is the essential requirement for fertilization. What follows is determined entirely by the nature of the egg itself.

The recent work of F. R. Lillie²⁴ reinforces still further this general point of view. His results indicate that in fertilization a union of a specific amboceptor-like substance, contained in the egg-cortex, with some other specific substance, also furnished by the egg, forms the primary or determinative event. The spermatozoön acts by removing the hindrance to this interaction, but other agents may act similarly—hence the possibility of parthenogenetic fertilization. This conception makes it clear why the presence of the sperm is unnecessary to the activation of the egg, and suggests that in its activating capacity this structure plays essentially the part of a specific releasing mechanism, in a manner which is thus closely analogous to that of a stimulus, as already indicated. The ensuing developmental processes are specific to the egg under investigation and require special analysis in each case.

SUMMARY

The chief experimental results and general conclusions of this paper may be briefly summarized as follows:

1. The action of pure isotonic solutions of neutral salts (0.55 m KCNS, NaI) in inducing formation of fertilization-membranes and cleavage in the unfertilized eggs of *Arbacia* may be prevented by anesthetics as well as by calcium and magnesium salts. The effective concentrations are those which just suffice to prevent cleavage in fertilized eggs.

2. The anesthetics are less effective than calcium or magnesium, and vary characteristically in effectiveness. The monohydric alcohols of the aliphatic series are the most favorable of those tried; the order of relative favorability runs: *i*-amyl > *n*-butyl > *n*-propyl and capryl > ethyl. Phenyl and ethyl urethanes have comparatively slight action, and chloral hydrate still less. Cyanide is ineffective.

²⁴ Cf. *Science*, N. S., 1913, vol. 38, p. 524.

3. The anesthetics have no inhibiting influence on the cleavage-initiating action of fatty acids. Since fatty acids readily penetrate the unaltered plasma-membrane, while salts do not, and since both agents are equally effective in inducing parthenogenesis, this difference indicates that the parthenogenetic agent acts at some point within the most external layer of the egg.

4. The favorable effects of after-treatment with hypertonic sea-water are prevented by anaesthetics as well as by cyanide. This result indicates that hypertonic sea-water, as well as anesthetics, acts by modifying the condition of the plasma-membrane.

OLFACTORY REACTIONS IN AMPHIBIANS

JONATHAN RISSE

ONE FIGURE

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INTRODUCTION

It is the purpose of this paper to record some observations and experiments on the olfactory reactions in certain amphibians, more especially in the toad (*Bufo americanus* LeConte). Initial experiments were tried on frogs (*Rana virescens* and *R. catesbiana*), both larval and adult, but the work did not prove promising and was therefore given up.

These investigations were undertaken at the suggestion of Dr. G. H. Parker of the Zoölogical Laboratory of Harvard College and to him for his kindly interest and helpful advice I wish to express my deepest appreciation.

FOOD AND ODORS

1. Relation of odors to food

There is little evidence that the relation of odors to food has been taken into account in any investigation of the habits of the different amphibians. In the quantitative studies of the stomach contents of frogs and toads no evidence has been found to show that certain foods are preferred by these animals. Fischer-Sigwart ('97) believed that frogs and toads were indiscriminate feeders. Needham ('05) has shown the food of the bullfrog (*R. catesbiana*) to be extremely varied. Lockwood ('83) in speaking of the toad, says "I do not believe it can smell. It catches insects, but only when such probable food is in motion." Knauer ('75), however, mentions cases where decomposing animal food was rejected by toads after having been taken into the mouth. Schaeffer ('11) found that certain caterpillars were refused by frogs in a similar manner. In both cases other factors may have been of disturbing influence. Hartman ('06) found no special preponderance of one species over another in the insects taken from the stomach of toads collected at random. Garman ('92) has records of the food of toads from which one might conclude that ants were more sought after than other insects. Such a condition, however, may have been due to the fact that younger newly metamorphosed toads being close to the ground met ants more frequently than they did

other insects. Hodge ('98) refers to the large numbers of houseflies eaten by the toad. Schaeffer ('11) mentions the variety of insect food taken by frogs in confinement. Quaintance and Brues ('05) show how toads make use of very diverse forms of insects as food. Slonaker ('00) and others have fed meat to toads, by simulating the motion of small insects to attract attention. The behavior of toads and frogs in confinement leads to the inference that food ordinarily must be in a living condition and in motion to be attractive.

Among food materials taken by the toad under normal circumstances there are many insects with characteristic odors. Conradi ('01) infers that the odor of the cucumber beetle (*Anasa tristis*) is inimical to the toad, but Hill ('73) observed no disastrous results to toads that had fed on this insect. Neither frogs nor toads hesitate to make use of other vertebrates as food, when occasion offers, or to devour members of their own species. Can it be shown that these animals are stimulated to seek for food or refuse it because of odors? With this question in mind the following feeding experiments were carried on with the toad (*Bufo americanus* LeConte).

2. *Materials and methods*

The toads used in these experiments were obtained in the vicinity of Cambridge, Massachusetts, quite late in the year. They were kept in a large box containing soil and leafmold in a moderately cool basement room. The soil was kept damp and the box dark. Some of the animals buried themselves in the soil, others took shelter under bits of wood. The animals reacted normally in all respects, taking food when offered. The food consisted mainly of mealworm larvae (*Tenebrio molitor*), of earthworms and dungworms (*Allolobophora foetida*), flies and other insects. Some of the toads were removed to large jars for greater convenience. Not all the animals were alike, some showing a greater tendency to hibernate than others. Food was generally given the toads at intervals of several days, but this procedure was modified as necessary. For convenience the experiments were carried on with the

toads confined in a large metal pan. In this way the animals were kept within bounds but were still allowed much freedom of movement.

3. Experiments

The first experiment was made to determine whether choice was made between two forms of food with specific odors. Previous to the trials earthworms had been fed freely to the toads, and it might be supposed that because of the peculiar odor of the dungworm, the latter would be refused as food.

Toads No. 1 and No. 2 were in the box in which the experiments were conducted before the dungworms were introduced. Toad No. 1 saw the worms soon after they had begun to crawl. It quickly hopped toward the worms and attempted to snap up the nearest worm. The attempt was unsuccessful and the lips were wiped with the forefeet. Sandgrains on the lips evidently produced a mechanical stimulus which was followed by the wiping.

The dungworms were then freed of all the adhering sand and again placed in the center of the box. Although the movement of the worms was sufficient to attract the toads, no further attempt was made to take the worms. When a mealworm larva was put in with the dungworms, it was quickly snapped up by the nearer toad, No. 1.

Dividing a dungworm then into a number of pieces, these were placed in a shallow vessel before the toads. Into the vessel there were also dropped two mealworm larvae. From the mass of wriggling pieces one of the mealworms was immediately selected and swallowed. Upon the dorsal surface of the other mealworm a drop of oil of pennyroyal was now placed; the mealworm with the oil was also quickly taken. Toad No. 2 made several unsuccessful attempts to take the remaining dungworm, but abandoned the dungworm, taking later some mealworms that had been put in the box. Similar trials with two other toads gave like results, leading to the conclusion that the dungworm is not used as food under normal condition, a conclusion that was subsequently shown to be false.

Some days later, the same toads, Nos. 1 and 2, were again tested with the dungworms. Three worms of medium size placed in the center of the box were immediately noticed by Toad No. 1. No attempts to take any worms followed. The odor characteristic of the worms was evident and the cutaneous exudate was visible. A mealworm dropped into the vessel among the dungworms quickly became covered with the slime of the worms. That the mealworm was recognized as a new object by the toad, is proven by the actions of the toad. The attitude peculiar to the animals when watching some object, was immediately assumed. As soon as the mealworm had crept away from the dungworms, the toad snapped it up. Another mealworm dropped into a dish containing some fragments of dungworm was watched by the toad as long as it moved. After it ceased moving it was of no more interest to the toad. No attention was paid to the fragments of dungworm although they were wriggling. A second mealworm smeared with the exudate from the earthworm was quickly snapped up by No. 1. This reaction was followed by the wiping of the lips with the forefeet; whether this was done to remove the slime adhering to the lips was not determined. The wiping action again took place when the same toad took another mealworm similarly prepared. Toad No. 2 exhibited the same reactions under similar conditions.

A series of trials with the different toads proved that fragmented dungworms were never taken, even though they exhibited decided movements. The trials also showed that the toads preferred the mealworms, and that the odor of the dungworms when applied to the mealworms was not deterrent to the toads.

Trials carried out with the same toads, to determine their preference for mealworms over earthworms, indicated that the mealworms were the more desired form of food. These were selected from among the earthworms. It is not clear why this should have been so; previously and later in the course of the experiments both earthworms and mealworms were taken promiscuously.

In a subsequent trial with Toad No. 1 mealworms placed among dungworms were taken after they had moved away from the dungworms. Broken pieces of dungworm taken into the mouth were followed by the wiping action. The exudate alone did not affect the toad in a similar manner. Although the odor was evident on mealworms smeared with the exudate, this did not hinder them from being taken, the only effect being a peculiar 'gaping' action after the mealworm had been swallowed.

To determine whether the color of the mealworm was a deciding factor, attempts to approximate the color of earthworms and dungworms were made. Mealworms tinted to resemble the color of the earthworms were taken without hesitancy. Such mealworms were always taken from among fragments of dungworms and earthworms.

In some trials for determining the effect of distinctly abnormal odors and natural foods, the following observations were recorded.

The toads experimented upon were No. 3, which was very responsive, and No. 4, which was sluggish. Dungworms were put in the experimental cage in a Petri dish. They attracted the attention of Toad No. 3, but otherwise it did not respond. The dungworms were then cut up and again placed in the cage, but again the toads did not react. Mealworms were now substituted for the dungworms and No. 3 immediately took two of these. Oil of pennyroyal was put on certain mealworms and when these were introduced, two were taken by Toad No. 3. No discomfort was shown by the toad. Fragments of dungworms were placed in the cage, but were not taken by the toads. Mealworms covered with dungworm juice were quickly taken, and accidentally one piece of a dungworm was taken and swallowed; another piece was brushed from the mouth. The mouth was opened several times in succession as though the fragment of worm had been unpalatable.

Four days later the same toads took mealworms with the juice of dungworms, and the exudate smeared upon them. Both toads took mealworms smeared with oil of pennyroyal unhesitatingly. Again later, the same toad, No. 3, did not discrimi-

nate between earthworms and dungworms. The latter were irritated in such a manner as to make the integumentary exudate distinctly noticeable. This was not deterrent in any case.

Whatever may have been the cause of the refusal of the dungworms in the earlier trials has not been made clear, for later trials showed no aversion to the dungworms on the part of the toads.

A series of trials with the toads, in which the conditions of feeding were carried to a degree far beyond what might be expected to occur at any time normally, showed that many odors were not repellent when associated with food.

To hungry toads was given the choice of taking mealworms with unknown odorous substances, and others without such substances. Ether, chloroform and alcohol could not be made use of, because of their fatal action on the mealworms. Clove oil, oil of cedar, oil of pennyroyal, oil of bergamot, oil of citronelle, aniline oil, carbon bisulphide and iodine in saturated solutions were made use of. In the experiments there often arose the question of a fatal dose. But none of the toads died during the trials. Several of the trial records are transcribed:

Toad No. 1. One mealworm eaten, after which two mealworms with oil of pennyroyal taken within one minute; only effect was 'gaping' several times. Rest for ten minutes, then mealworms put in a dish too high for toad to get into. Toad in attentive attitude, and again when offered took clean worms and also one with oil of cloves. Odor was not repellent, nor was any after-effect noticed.

Four days later the same toad ate three mealworms, two with clove oil and one with oil of citronelle.

Toad No. 3. Took in two successive trials, two clean mealworms, two with oil of pennyroyal and two with carbon bisulphide; and later two clean mealworms, two with aniline oil, and two with oil of rose geranium.

Toad No. 5. At one time, took two mealworms with oil of pennyroyal and one earthworm with the same oil applied.

The oil of pennyroyal appeared to be more irritating to the lips of the toads than the other substances used; the wiping

of the lips was the only reaction seen after the toad swallowed worms treated with this oil.

Iodine, when taken into the mouth with the larvae seemed to be more productive of discomfort than the oils did. The larvae were not refused at any time during the trials, nor were the substances deterrent because of the attendant odors.

4. Experiments with non-living food

Some experiments were made to determine whether animal food that gave no evidence of being alive would be taken by the toad. In the feeding trials previous, only mealworms in motion were used for food; no attempts to use inert worms were made. Pupae of the mealworms were offered the toads. When the pupae were first dropped into the cage, they often made spasmodic motions, but these soon ceased. Sometimes the toads took the pupae when thus in motion. Mealworm larvae, freshly killed and motionless, were never taken. By means of a delicate strand of silk threaded into a larvae, these could be dragged over the bottom of the cage in a manner to imitate partially the living condition. The toads could be induced to snap up such larvae, and when once in the mouth they were not rejected. Knauer ('75) gives instances of the rejection of decomposing earthworm. My experience was to the contrary. Bits of meat fashioned into semblance of mealworms when thus drawn past the toad, and taken into the mouth, were not rejected.

Dead flies also could be suspended and moved before the toads. These were attractive to the toad even if abnormally odorous. Artificial larvae were fed in a few instances to the toads. These were made from absorbent cotton and paraffine, and could be substituted for the living worms if set in motion by means of the thread.

Toad No. 1 swallowed two such false larvae in the course of one trial. These false larva were smeared with oil of clove and iodine solution in different trials.

In the presence of the living larvae, no attention was paid to motionless artificial larvae, but if these were set in motion they were sometimes taken.

Effects of the substances used in these experiments may be summed up as follows: The odor of the iodine solution was not deterrent in any manner; the after-effect seemed to be more disturbing in the mouth region than did that of other substances. Oil of pennyroyal and oil of rose geranium when they touched the lips led to wiping the mouth-parts with the forefeet. No similar effect was noticed from clove oil, cedar oil, and bergamot oil. When bisulphide of carbon was applied to the mealworms, the toads gave some evidence of discomfort, gulping several times in succession after having taken larvae prepared with this substance. Aniline oil did not cause any reactions that seemed to result from stimulation of the mouth-parts, nor was it repellent because of its odor.

FOOD AND DARKNESS

The diurnal retreat of the toad into dimly illuminated places and its habit of feeding in the night are well-known. Frogs on the contrary are most active during the daytime. In connection with the feeding experiments on the toad it was desirable to know what degree of darkness was prohibitive to its finding food. Are other stimuli present, do smell, hearing and touch share in the reactions of seeking food?

1. Materials

The same individuals that were used in the previous experiments were used in these trials. The toads appeared to be in normal condition, giving no evidence of having undergone any untoward experiences. No changes were made in the care given the toads. The experimentation was done in a photographic dark-room with controllable illumination.

The experimentation chamber was a box, 30 inches long, 20 inches wide, and 8 inches high. Loam and leaves had been allowed to remain in the box for some days, thus giving the chamber some semblance to the toad's natural habitat.

2. Experiments

To ascertain whether any stimulus other than the optic is called into play in the finding and taking of food the following experiments were performed.

Toad No. 1 was placed in the box, which was empty except for a covering of moist filter paper on the floor, and allowed to remain undisturbed for some time. There was no excited hopping about during this period of illumination. The light having been cut off, the toad crept along the wall, and finally came to rest in a corner where it attempted to burrow as though it were on soil. After two hours of darkness some mealworm larvae were introduced into the box. These were placed centrally on the floor and as these changes were made in darkness the larvae were not seen by the toad. The toad was then lifted from its position in the corner of the box and placed immediately over the glass plate on which the larvae were and facing them. In this position there was no thigmotactic stimulation, as there would have been in the corner position. By listening to the sounds within the box the movements of the toad could be followed. The position of the larvae and the toad were noted at the end of two minutes of darkness. The toad had resumed the corner position, the larvae had moved out and away from the plate, one of them being within 2 inches of the toad. Two minutes of darkness brought no further movement on the part of the toad. The larvae were distributed around the sides of the box. After another minute of darkness, the light was turned on and at the first visible movement of the mealworms, the toad turned and jumped toward them. There followed no movements on the part of the toad in successive periods of darkness; but each time the box was illuminated the least movement on the part of the mealworms called forth characteristic movements from the toad.

Toad No. 2 was tried with dungworms in a manner similar to that just described for Toad No. 1. No notice was paid to the worms in the dark but in the light the toad followed the

worm some distance and finally picked it up, using both the tongue and lips to do so.

In another trial, the toad was placed in the center of the floor and the worms were distributed in the portions of the box near the walls. The illumination was lateral, thus leaving one side of the box quite dark. Movement of worms in the darker regions of the box were not seen by the toad, even when it was turned toward the worms. When a worm moved in the light part of the box, the movement soon attracted the attention of the toad and the characteristic reaction followed immediately. Repetition of the experiment several times with other toads gave the same results.

When the dungworms were replaced by pillbugs (*Oniscus*), these were immediately caught when in the light, but not when in the dark.

Other trials with mealworms, earthworms and dungworms gave like results; only during the periods of illumination was the food sought and then with no discrimination in favor of any particular form.

Other trials, with very dim daylight as a means of illuminating the chamber gave identical results. If the mealworms or the dungworms were in the darker portions of the chamber, they were unnoticed by the toad. When the box was covered so as to cut off all illumination, there were no movements on the part of the toad; darkness prohibiting entirely any food seeking activities.

In the lighted portions of the chamber the worms were only seen if in the direct visual field; if somewhat to the rear or to the side of the toads they apparently could not be seen, except when in very vigorous motions.

3. Abnormal odors and darkness

In connection with the darkness experiments, trials were made with the same odorous substances that had been used in the preceding experiments. The same individual toads were used as before. Mealworms were treated with applications of oil of clove, carbon bisulphide, and oil of rose gera-

nium and were allowed to remain in the experimental chamber with the toads under the same light conditions as described in the preceding trials. The presence of the odors did not stimulate the toads to seek or avoid the worms in the dark. Again, as observed in the previous trials, the presence of the odorous substance did not deter the toads from taking food in the light.

ODOR-STREAM EXPERIMENTS

1. Apparatus

From the experiments previously recorded there was no positive evidence that odors were concerned with the taking of food by the toads. Odorous substances when taken into the mouth by accident do not stimulate the receptor organs to the degree of inhibiting the act of deglutition. A possible explanation for this might be sought in the relation of the external nostrils to the mouth. Even if the olfactory function were present to but a slight degree, it might be possible to demonstrate its presence by leading the odors directly to the nasal opening. If the vapors could be brought directly to the epithelial surfaces in appropriate manner, reactions might occur. It seemed desirable to devise some method for doing this.

After some trials, an apparatus of a satisfactory kind was finally devised. An outline of it is shown in figure 1. The arrangement of the parts is as follows: From the reservoir, *A*, water displaced the air contained in *B*. By means of screw clamps at *x* and *y* the rate of flow of the water was controlled. The clamp, *x*, was adjusted for the flow, while clamp *y* is used for starting and stopping the stream as a whole. *C* is a small reservoir in which was suspended a vial, *D*, containing the odorous substance used. The distal end of the outlet tube, *b*, was submerged in the material contained in *D*; the air stream carried over and out of *C* would consequently be impregnated with the odor to be tested. By means of appropriate connections and the nozzle tube, *d*, three millimeters in diameter and bent in proper form, the air stream was led into the experiment chamber, *E*. Elastic suspension of the tube, *d*, automatically raised the tube

and allowed freedom of movement. For control tests there was provided a duplicate of the tube, *d*, which could be connected directly with *B*; thus avoiding any possible errors by contamination from the material in *D*. The chamber, *E*, was an open cylinder, the lower end resting in a shallow vessel containing soil easily changed and moistened. To eliminate other dis-

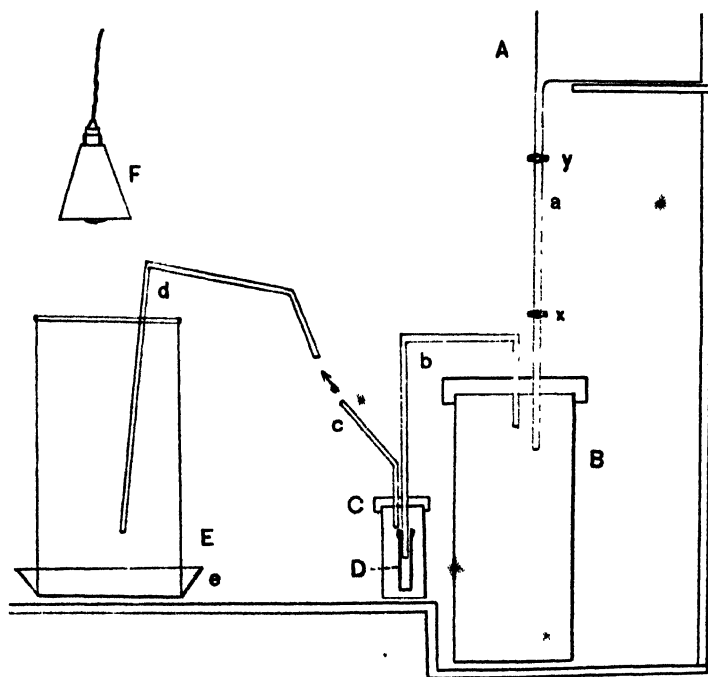


Fig. 1 A, reservoir; B, air chamber; C, odor chamber; D, vial; E, experiment chamber; F, lamp; a, water tube A to B; b, airtube B to C; c, outlet tube for odor stream; d, nozzle tube for chamber E; e, vessel containing sand; x, y, clamps.

turbing factors the cylinder was covered with black cloth. The illumination of the chamber, *E*, was by an electric lamp of 8 c.p. This was of advantage in orientation, as the toads are positively phototropic (Pearse '10).

The apparatus was arranged near the water supply in a basement room from which daylight could be excluded. Several other chambers similar to *E* were provided in order to adjust

the toads to the new conditions. Toads not accustomed to the chamber were at first much disturbed, reacting to various other influences. Attempts to crawl out of the chamber, to bite the end of the tube, *d*, or to be restless within the chamber were some of the reactions noted. After adjustment normal reactions were then exhibited, such as feeding or burrowing into the soil. The apparatus was kept well ventilated between periods of experimentation as well as during the testing periods.

2. Rate of flow of odor-stream

The rate of flow of the air stream was made as uniform as possible. If allowed to flow uninterruptedly the 3000 cubic centimeters of air in *B* were displaced by the water from *A* in from 80 to 90 minutes. At this rate the flow is approximately 35 cubic centimeters per minute. That such a stream is not sufficiently strong to be perceptible can be demonstrated by placing the nozzle tube near the moistened lips or the tongue. The flow of the stream could also be judged from the frequency of escaping bubbles when the end of the tube was immersed in water.

The tests as carried out followed a uniform plan consisting of, first, a control test with air, and secondly, a trial proper with the odor stream.

3. Methods

In the control test the toads were first subjected to the air stream coming from *B*. The nozzle tube, *d*, was directed toward various body regions, namely, flank, axillary region, anal region, and surface of the eye (as near as possible without contact). After this test for possible stimulation of integumentary sense organs, the air stream was next directed to the nostril to ascertain if there was any stimulation of the nasal epithelium. The test with the odor stream followed next and the same parts of the body were tested as before.

The toads used in the feeding experiments were used for the tests with the odor stream and were normal in all their activities.

The one exception was Toad No. 4, which seemed to be in a semi-hibernating condition during the entire period. Two other toads taken in the fall of 1912 were likewise tested with this apparatus. Although no two individuals were identical, the results of the trials agree in all essentials.

4. Substances used in the tests

Oil of cloves, oil of pennyroyal, oil of rose geranium, cedar oil, bergamot oil, aniline oil, carbolic acid, olive oil, castor oil, and cod liver oil were used, though some of the substances gave entirely negative results.

Trials were also made with the odor stream from food materials used by the toad. For these tests mealworm larvae, earthworms, dungworms, and cockroaches and other insects were put directly into the chamber, C and the air stream was allowed to carry over any odors that were present. In no case was there evidence of olfactory stimulation from these bodies.

5. Experiments

The attempt of Graber ('85) to determine the olfactory reactions in some of the amphibians proved unsatisfactory. Aronsohn ('86) and Gourewitsch ('83) noted the effect of odors on the rate of respiration. The animals, while confined in covered beakers, were exposed to odors of turpentine and eau de cologne with disturbing results.

Early in my own tests it was seen that chloroform, ammonia, ether, turpentine, formol or alcohol when introduced into the chamber were disturbing. Even though the air stream with these substances was not near the nostril, reactions followed very quickly and with the proximity of the stream to the nose the disturbance was increased.

Ammonia, chloroform and turpentine when directed upon the anterior head region induced the toad to jump away from the tube to one side or even out of the chamber. The odor of turpentine resulted in a very characteristic attitude, the head

being bent down between the forelegs, the body raised from the ground and best described as humpbacked. Ether or alcohol did not produce reactions so decidedly vigorous, though the toads retreated quickly from the tube.

The respiratory movement was retarded by all the substances that stimulated the olfactory end-organ. Tests made with the odor stream when the act of inspiration was suppressed were devoid of any resultant motor activity.

After the earlier tests with ammonia, ether, etc., the essential oils were used and some were found to be more effective in calling forth characteristic reactions than others. Some were entirely negative in effect, as, for instance, castor oil, cod liver oil, and olive oil.

What reactions could be considered as directly called out by the stimulation of the olfactory organ?

When the odor stream from oil of cloves or pennyroyal was allowed to spread over the anterior region of the head and presumably enter into the nasal cavity with the inspired air, the first motor act consisted of a slight bending down of the head, away from the nozzle of the tube, and a cessation of the respiratory movements. If the tube were removed quickly, the toads soon resumed the normal position and the respiration movements went on again with little interruption. If the odor stream was allowed to flow continuously upon the nostrils, respiration was entirely suppressed for some time. Often the toad made motions with the forelegs resembling wiping. When very much stimulated by the stream the animals moved away from the tube. The bending of the head was most noticeable when the toads were partly hidden in the sand. At such times respiration ceased, the head was bent more anteriorly and the animals endeavored to burrow down into the soil. The act of burrowing could be hastened by directing the tube repeatedly at the nostrils during such a test. This position of being partly buried in the soil was most favorable for observation. When thus buried the animals did not move about and the stream could be directed more accurately against the nostrils than at other times when the animals were free to move about.

The tests were carried out in periods of approximately five-minute durations, followed by intervals of rest varying in length of time. It sometimes happened that the animals were too restless to allow a fair interpretation of their reactions; in such cases the tests were discontinued. Other individuals again, were amenable to the trials for several hours without showing themselves to be disturbed. Precaution was taken that no odors entered the chamber otherwise than by means of the appropriate tube.

Records of the trials indicated what reaction followed the stimulus applied to the nostrils. The records shown in table 1 will serve as samples.

TABLE 1
Toad No. 1

TIME	STIMULUS	REACTION
3.00	air	moved to one side
3.05	repeated	burrowing normally
	toad was allowed to rest fifteen minutes	
3.20	odor of oil of clove	head bent downward
	repeated	head bent downward
	repeated	wiped with right foot
	repeated	moved away to left
	repeated immediately	moved away to left
	repeated	wiped; attempted to climb
3.25	repeated	wiped again

Toad No. 2

10.30	air	at rest
	repeated	at rest
	repeated	at rest
10.35	repeated	at rest
	Toad allowed to rest five minutes	
10.40	odor of oil of pennyroyal	wiped
	repeated	head bent downward
	repeated immediately	head bent downward
	repeated	moved toward right
	repeated	head bent downward
	repeated immediately	head bent downward
10.45	repeated	head bent downward

Bergamot and cedar oil resulted in fewer reactions than oil of clove and oil of pennyroyal, showing them to be less stimulating than the other oils.

As already indicated, the stimulus was effective only while inspiration was in progress, for pressure of the stream was so slight that it could not force the odorous particles into the nostrils. The reactions were not characteristic of the different odors. It might be said that the reactions differed in degree, only, since there were only differences of vigor with which the reactions were executed. Even individuals differed in this respect at different times during the trial periods. Other factors such as temperature or tendency to hibernate may have had some influence in this aspect of the reactions. Absolute parity of the tests could of course not be obtained.

The relative effectiveness of the odors in stimulating the receptors was as follows: Most effective and approximately equal were oil of cloves, and oil of pennyroyal; less effective were oil of rose geranium and cedar oil; and least effective, with reactions infrequent, were bergamot oil and carbolic acid.

Odors from castor oil, olive oil, cod liver oil, living mealworms, earthworms, dungworms, cockroaches, decaying meat and decaying leaves in soil were without effect.

6. Controls and operations

The controls used in the tests seemed to be confirmatory of the presence of the olfactory function; yet a possibility existed that other receptors had been stimulated at the same time. The ophthalmic branch of the trigeminal nerve supplies the region of the head anterior of the nostrils, with some fibers possibly present within the nasal capsule. This being so, stimuli coming to this region of the head could call forth reactions easily through this branch as well as through the olfactory organ. To ascertain whether this branch had been stimulated several sets of experiments were tried.

The nostrils were closed by suturing with silk thread. Closing the nostril in this manner was of more serious consequence to the

toads than to fishes. This established an effective hindrance to the respiratory current, therefore it was found impracticable. If the stitches were not placed deeply, the muscular movements of the nostril soon caused the thread to cut through the margins. Furthermore, complete closure of the nostrils was apparently fatal in several preliminary trials.

Filling the nasal aperture with melted vaseline was only partly successful, entrance to the capsule being prevented by the nostril valves. The vaseline could be "troweled" into the opening. It was evident from the actions of the animals that the vaseline was discomforting. The toads showed decided restlessness and attempted to wipe their heads.

Some tests were made with toads so treated. When both nostrils had been successfully closed, no reactions traceable to odorous substances could be observed. If only one of the nostrils was closed, the odorstream, when directed upon the open nostril, was effective in causing a reaction.

Clove oil and pennyroyal were the substances used in the tests with the nostrils partly or wholly closed, these having been most efficient in calling forth reactions in the normal toads.

To be certain of the assumption that the reactions noticed with the odorstream were called forth by the stimulation of the olfactory receptor, operations of two kinds were performed on the toads.

In one of the operations, the olfactory tract was severed. In the other, it was necessary to section the ophthalmic branch of the fifth nerve. After etherization, the olfactory tracts were severed at the anterior border of the eyeball. The ophthalmic branch of the trigeminal was cut by piercing the integument of the optic capsule at the anterior inner angle of the orbit and cutting across the floor of the orbit. After recovery, only the specimens reacting normally to other stimuli were used for the tests.

Toads in which the olfactory tracts had been severed did not react to the odorstream. If partly buried in the sand, they were undisturbed by odors coming through the tube. The head was not drawn down, nor did they move away from the tube. One

EXPERIMENTS WITH TADPOLES

1. General

Knauer ('75) emphasizes the fact that some of the larval anurans are less phytophagous than is commonly supposed and instances the fondness of toad tadpoles for animal food. Holmes ('07) also states that decomposing insects, earthworms, etc., are acceptable as food to frog tadpoles. That certain European anurans feed indiscriminately like the earthworm is a well-known fact. Whether vegetable material must not be in the early stages of decomposition before becoming available as food for tadpoles has not yet been determined. It is certain that many unicellular and filamentous algae may be ingested repeatedly before digestion is complete.

Nagel ('94) denied the olfactory function to aquatic vertebrates. His theoretical objections have been refuted for the fishes by the work of Parker ('10; '11), Sheldon ('11), and Cope-land ('12). The question still remains open as regards amphibians and its solution depends upon suitable material and methods. This was reason sufficient to attempt an investigation of the olfactory function in the tadpole of the toad. Preliminary trials gave evidence at once that the tadpoles were responsive to the presence of decaying animal matter in their immediate vicinity. Tadpoles placed in a vessel containing filtered water for a day or two, the feces being removed frequently, soon come to be in a state of hunger. Water not filtered contained at all times organic material enough to form a delicate film upon the walls of the vessel. This film of partly decomposing organic matter is a source of food for the tadpoles and must be removed. For like reasons the feces must be taken away.

In the preliminary trials particles of dead earthworms, dead fish or bits of meat undergoing decomposition were placed in the vessel containing the hungry tadpoles. Such particles of food were quickly found by the tadpoles.

The feeding trials were performed on tadpoles of three successive years. The first set was obtained at Woods Hole, Massachusetts, on August 10, when the tadpoles were about to

metamorphose. Some of the larvae had one, others both pairs of appendages well developed, and a few specimens passed through the final phases after having been brought into the laboratory. They were kept in shallow vessels with some of the bottom detritus from their original habitat. Experimentation and natural causes gradually diminished the numbers available for the trials, but the tadpoles appeared to be normal in their activities at all times. The tadpoles surviving through the period of experimentation were killed by accident in the month of January following.

The second set were tadpoles taken in May of the following year in the vicinity of Cambridge. They did not lend themselves well to experimentation. Conditions appeared to be unfavorable for them in the laboratory. Experiments therefore were carried on with but small numbers, but so far as these went they were corroborative of those from the first set.

The tadpoles included in the third set, were taken in June, 1913, from two different localities: namely, Cambridge and Woods Hole, Massachusetts. The tadpoles were experimented with particularly to show that the olfactory reactions might be completely checked by a certain procedure and then revived.

2. Methods

The method of procedure in the trials was as follows: The tadpoles were placed in filtered water for twenty-four to forty-eight hours before experimentation began. Then the food was introduced into the vessel; the reactions being noted in accordance with the shifting of the food placed in the vessel.

Food used in the tests consisted of particles of fish, earthworm, or meat in decomposition. At first the food without any envelope was placed free in the vessel, but later it was wrapped in cloth. In other trials again, two packets were placed in the water; one containing the food, the other without it.

Very little difficulty was experienced in noting the reactions of the tadpoles. When the two packets were presented, the tadpoles distinguished quickly the one containing the food.

When confined to shallow vessels, the actions of the tadpoles of the toad and of the frog of approximately the same stage, were somewhat different. In general, the frog tadpoles swim along the vessel walls. The toad tadpoles, on the other hand, move in all directions over the bottom or near the upper surface of the water.

When toad tadpoles came into the vicinity of the food, either covered or open, the reactions seemed to indicate stimulation of some kind. They swam from side to side, and often when near the food would turn directly to it. When the food was found the tadpoles attacked it very eagerly. When two packets had been introduced the tadpoles did not remain upon the packet without food nor did they nibble it as they did the one containing food.

3. Experiments

a. First set of tadpoles. The records of several of the trials are here given in detail:

TIME

- 8.20 Food packet placed in shallow dish containing eight tadpoles
- 8.22 Food found by first tadpole
- 8.23 Food found by second tadpole
- 8.39 Food found by six tadpoles
- 8.50 The position of the food was changed
- 8.55 The food found by three tadpoles
- 8.57 The food found by five tadpoles
- 8.05 The food found by seven tadpoles
- In another trial a small piece of fish not covered was placed in the dish.
- 10.30 Food placed in dish containing eight tadpoles
- 10.40 Food found by six tadpoles
- In another trial two packets were placed in the dish, one with food, the other without.
- 2.35 Food placed in dish with six tadpoles
- 2.43 All the tadpoles had found the food and were nibbling
- None of the tadpoles were at the false packet.
- 2.55 Reversed position of the two packets
- 3.20 Food found by five tadpoles
- In a similar trial with two packets, one containing fresh meat, the other without.
- 10.42 Packets placed in dish with fifteen tadpoles
- 10.50 Food found by twelve tadpoles
- 10.56 Reversed position of packets
- 11.05 Food packet found by ten tadpoles

The totals of the trials carried on with the larva of the first set gave these results: In 160 trials, food packet was found 120 times. Trials carried on from time to time up to the accidental loss of the tadpoles resulted in similar ratios.

b. Second set of tadpoles. The conditions were unfavorable for keeping these tadpoles and because of this circumstance only a small number of individuals were available. The trials were carried out in a manner similar to those for the first set.

It needs only be said that the results were similar to those obtained from the experiments of the first set. Precautions were taken with this set to have the two packets identical in appearance, and to transpose them in position. To avoid the accidental finding of the packets as much as possible, they were placed some distance from the sides of the vessel. The toad tadpoles swam more rapidly than the frog tadpoles, and were also more erratic in their movement in the water. It could be easily determined whether they were influenced by the presence of the food mass near them. In a few cases only did there seem to be a visual stimulus influencing the tadpoles to react; such being occasioned by the very lively actions of other tadpoles already at work on the food mass.

c. Third set of tadpoles. For the purpose of verifying the results of the previous experiments, similar experiments were performed with the tadpoles of the third set in June and July of the third season. As stated, these tadpoles were obtained from two different localities and kept successfully in the laboratory, metamorphosis being deferred for the time being. The tadpoles were unquestionably those of *Bufo americanus*, having been obtained earlier than the time when the mating call of Fowler's toad was heard.

The food used in these experiments was dead and partly decomposed frog tadpoles, earthworms, and beef liver. These materials were either placed freely in the vessel or covered with cheesecloth one or two layers in thickness. In a similar way, as previously described, two packets were used in some of the trials; one containing the food, the other being identical in appearance but without food.

A number of trials in which the food was placed in the vessel with the tadpoles showed that the reactions of the tadpoles depended somewhat on the size of the vessel. In a small vessel the time necessary for the food to be found by a certain number of the tadpoles was usually less than in a larger vessel. The same is true if the food is placed near the side of the vessel, say within an inch of the side wall; in such a case the animal swimming through the zone impregnated by the odor is influenced more quickly than when the food is centrally placed.

In the trials with two packets discrimination was very evident in the actions of the tadpoles. In the beginning of the trial the reactions were at all times apparently without choice. After the packets had been allowed to remain in the water for a short time, the tadpoles always endeavored to feed from the packet containing the food. Even if the tadpoles rested on the 'dummy' packet very little attempt was made to nibble. Transposition of the two packets was accompanied by a corresponding redistribution of the tadpoles.

This last method was modified several times, by exchanging the envelopes of the packets, or by substituting an envelope saturated with the odor of the food material for the food itself. When this was done, the tadpoles congregated upon the food-saturated envelope, finding it as they did the food open in the water, or when contained in the envelope and allowed to remain for some time.

* An attempt was made to determine whether the tadpoles would orient themselves to an odor-saturated waterstream which was allowed to flow into a vessel containing them; but this experiment was fruitless of results, although there was some slight evidence that the current was especially stimulating.

Corroborative of the earlier findings of the experiments on the two previous sets, experiments carried out with the third set were of greater value in this study in what may be called operative tests.

To determine whether the reactions of the toad tadpoles as already described are due solely to the stimulus received

by the olfactory organ, attempts were made to inhibit such stimulation in various ways. This was found to be rather difficult and in the light of the earlier attempts the evidence was not very convincing. The diminutive size of the tadpoles is the principal factor militating against successful operations.

Cutting the olfactory tract is the method by which inhibition may be made absolutely certain. In this method the chief difficulty lies in determining the proper degree of anesthetization for the operation and subsequent revival of the animal. Chlore-tone of 0.1 per cent was used for this purpose, as the tadpoles could be brought into a vessel with fresh water and revived. After being anesthetized the tadpoles were bedded in a bit of absorbent cotton held in the hand, and with a needle the cranial case was pierced in the median line anterior to eyes. The shock attendant upon the operation or the manipulation was disastrous in most cases, and only a few specimens survived. Within a few days these tadpoles became less vigorous, and finally all succumbed.

The few individuals so operated upon and tested for reactions resulting from stimulation to the olfactory sense organ did not give sufficient evidence from which satisfactory conclusions could be drawn.

In another manner the inhibition of the stimulation and the reactions was also attempted. The external nares of the toad tadpole are comparatively large and it was possible to fill the nares with white vaseline, the tadpoles being bedded in moist absorbent cotton. Tadpoles so treated showed the presence of the vaseline to be disturbing in effect. The plugs prevent the respiratory stream from entering the nasal openings, the swimming movements are not so vigorous as under normal conditions, the chief endeavor of the tadpole being to free itself from the disturbing material. The temperature of the water is to be taken into account here, as the vaseline could be removed quite readily by placing the tadpoles in water slightly warmed. As soon as the vaseline has been dissolved the tadpoles again act in normal manner. This method of treatment was made use of in the first

and second sets, but on account of the comparatively small numbers at hand, the results could not be considered as conclusive.

With the material of the third set the chief aim was to determine whether the presence of the vaseline plugs was beyond doubt inhibitive of the apparent olfactory reactions. Some operations of cutting the tract were carried out, but the after effects were in most cases disastrous to the tadpoles, and therefore special stress was placed on plugging the nasal openings.

Making use of the same individual tadpoles in the successive trials, these groups were isolated in filtered water for some time previous to each trial, and tested repeatedly for reactions to the food as presented (without an envelope) while the nasal openings were in normal condition, or when filled with the vaseline plug.

The trials with the tadpoles in this series were carried out in the following manner: The tadpoles were isolated in filtered water for at least twenty-four hours, in a few cases forty-eight hours, but not beyond this, as the tadpoles showed that a longer period without food was disastrous to them. After isolation for the designated length of time, the tadpoles were tested for the presence of food in the water; immediately after this the nasal plugs were put in, and the tadpoles transferred to a vessel free of food or vaseline. The food was then introduced. Unless the food mass was placed immediately in the proximity to the tadpoles they exhibited no such activities as they previously had. When swimming and moving about in the vessel, there were no such positive attempts to find the food as when normal. The trials were repeated several times with different groups of individuals, in each case allowing several days to intervene between the trials, the tadpoles being provided with food and water from the stock.

The tadpoles showed no bad effects from the plugs of vaseline in the nasal openings, reacting freely in the later trials as well as in the earlier experiments.

Tests made with the tadpoles having the plugs in the nares showed them unable to discriminate between two packets one of which contained food, while previous to the plugging definite choice had been made by them in favor of the food packet.

The conclusion reached from these experiments as described for the different broods of tadpoles is that the presence of the nasal plug is effective in inhibiting any stimulus coming to the sense organ and consequently no corresponding reaction follows.

DISCUSSION

1. Toads

In its method of obtaining food the toad seems to respond to the visual stimulus entirely. This stimulus is apparently effective only when it involves motion. It is not always followed by perfect reaction, for substances inappropriate as food are often taken accidentally. Rejection of such material occurs in compliance with mechanical or tactile stimulation.

Nor does the gustatory function appear to be of any importance in feeding. Gaupp ('04) does not consider the function of the epithelial endplates of the mouth cavity to be established. He refers to Bethe as bringing forward the best evidence favoring them as tactile organs. It has been shown that strong solutions of picric or acetic acid applied to the epithelium of the mouth cavity will cause appropriate motor activity. Such reactions take place without reference to the point of application of the acid, whether this be in regions supplied with endplates or devoid of them. The latent period between stimulus and reaction is of appreciable duration.

These considerations speak against the belief that these organs are gustatory in function.

Until the contrary is proven, the similarity of food materials of frogs and toads argue for similarity in structure of the epithelium of the mouth. Observations and feeding experiments tend to confirm this view. Food does not remain in the mouth cavity any great length of time. Deglutition follows almost instantly, and therefore the sense of taste would be of minor importance. Under certain conditions regurgitation may occur. If substances disagreeable to taste are taken into the mouth the animals might be expected to resort to this expediency more often.

In no case in the course of the feeding experiments was cognizance taken of the unusual substances coming into the mouth to the degree that food was ejected or regurgitated. The experience cited by Knauer ('75) of toads refusing decomposing earthworms is probably referable to tactile stimulation. The nature of the organisms serving as food for the toads under natural conditions is such, that materials differing in texture markedly from the normal might be sufficient to cause refusal.

To establish any connection between food used by toads and the possible odors inherent to the food seems difficult at present. Although evidence is negative, this is qualified by the fact that the data are really not sufficient to establish any conclusion on this question. Natural foods are apparently taken indiscriminately. Stimulation of the receptors may take place; the presence of such stimulation and the effect are not yet demonstrable.

Our inability to recognize the quality of particular motor reactions following certain stimulations does not argue against the absence or refinement of reactions.

The experiments of Graber ('85) were such as to allow no great value to be attached to them. The unmodified methods used for forms differing so greatly in phylogenetic position and in habit with the very doubtful reactions as recorded, speak against the acceptance of his data as important.

To a similar degree the experiments of Aronsohn ('86) on odors and respiration are of little value in indicating the use of the olfactory organ in anurans. His experiment does not preclude stimulation of the trigeminal nerve. His choice of substances and the manner of experimentation favor the possibility that the fifth nerve is involved. The instance mentioned by Conradi ('01) has not had confirmation of any kind. In the experiments described, the presence of abnormal odorbearing substances has not given origin to stimulation sufficiently strong to inhibit the desire for food.

In all experiments carried out with the lower animals and their reactions toward solutions or vapors there exists the possibility that solutions or vapors are more dilute than was in-

tended. Whether stimulation is to take place in air or water, it is extremely difficult to work with solutions or gases absolutely standardized. The results of the experiments as conducted show that odors when in relation with food are not sufficiently deterrent in action to compel the toad to refuse such food. Odors of natural surroundings may stimulate the toads to certain reactions. At present there is no evidence that odors of soil or water are effective in any degree on the olfactory organ of the toad.

2. Tadpoles

In contrast with the tadpoles of the frog, the toad tadpole may be claimed to possess an olfactory sense, and possibly to a much greater degree than might be supposed. Anatomically considered the two species appear similar. Differences make themselves evident in a closer study of certain parts. The nasal openings in the toad tadpole are relatively larger than in the frog tadpole. The water stream into the nasal passage of the toad tadpole is therefore of greater magnitude than in the frog tadpole.

Contrary to Exner ('78), as quoted by Gaupp ('04), the nasal openings in both species serve for the incurrent water stream. If to this stream are added other substances: dilute solutions of methylene blue or particles of carmine, the toad tadpoles are very quick to respond to the stimulus. Such substances added to the water current flowing into the nasal chamber of the tadpoles of the frog (*Rana virescens* and *R. catesbiana*) produce similar reactions. Toad tadpoles react at the immediate entrance of the first substances into the nostril; tadpoles of the frog will permit the stream to flow into the nose for a long period, reacting much more slowly to the stimulus. Probably there is a mechanical stimulus from the carmine which sets free the response.

It is more than probable that toad tadpoles recognize certain foods and their odors. When given the choice as between decaying animal matter and decaying plant substances the former is preferred.

In the series of tests there was always positive reaction in the presence of the animal food when opportunity for such choice was given. Organs of taste have not been demonstrated in the mouth of the tadpole.

On the other hand, the differentiation of the nasal epithelium into the characteristic olfactory organs and the supporting structures takes place very early and we may safely assume the reactions noted to be the result of stimulation of the olfactory receptors.

3. Conclusion

The anurans have been called microsmatic animals; such distinction being based on histological and anatomical comparison with the animals classed as macrosmatic.

The nasal organ of the Anura is a common respiratory and olfactory organ, and in this respect conforms to the organ possessed by the higher vertebrates.

A chambered nasal cavity of considerable magnitude supplied extensively with olfactory epithelium and adequate connections with the central nervous system, predicate functional activity of the sense organ.

The life habits and the phylogenetic position of the anurans suggest that such functional activity not only be present but adequately developed. The presence of receptor organs identical in structure with those found in the higher vertebrates further postulates that functions peculiar to these structures be identical.

The receptor peculiar to the olfactory organ of all vertebrates consists of a neurone whose cell body is peripheral in position. The distal portion of the neurone is characterized by the protoplasmic processes projecting above the level of the surrounding cells, while the proximal end is attenuated and gives rise to one of the fibers of the olfactory nerve. This type of receptor is directly comparable structurally with neurone cells found in the epidermis of many invertebrates (Parker '12). Neurones such as these, found in some of the invertebrates are distinct portions of the receptor-effector system and have been demonstrated as extremely sensitive to chemical stimuli. That

such receptors, olfactory in function and responding only to stimuli when the cell surfaces are dry were peculiar only to air breathing animals was the earlier assumption.

The work of Aronsohn ('86) and Veress ('03) has shown that in man and the higher vertebrates the olfactory epithelium is bathed by glandular secretions and whatever stimulus reaches the receptor must do so in the form of solutions.

More recently Baglioni ('09), Parker ('10; '11), Sheldon ('11) and Copeland ('12) have demonstrated in aquatic animals the stimuli inducing certain reactions to be identical with those noted in the air-breathing animals.

If this assumption is valid, as it appears to be, that the olfactory receptor is the simplest and least differentiated of receptor neurones and stimulated by extremely dilute solutions, we should find the olfactory organ in *Anura* readily stimulated.

That well defined and characteristic motor reactions have not yet been recognized as results due to stimuli varying in quality may be due to lack of observational data and methods of experiment.

SUMMARY

1. There is no evidence that toads react to olfactory stimuli pertaining to soil, water, etc.

2. The character of the food is not differentiated by attendant odors to the degree that the adult toad thus distinguishes it.

3. Substances of unusual character and odors, when associated with food, do not stimulate the olfactory organs in such a manner as to bring the toad to refuse the food.

4. The presence of such substances in close proximity to the toad, and invisible because of darkness are not repellent in effect on the toad.

5. Odorstreams specific in character, made to flow over and into the nasal openings stimulate the olfactory sense-organ; such stimulation causing definite motor activities to follow.

6. Appropriate operations are confirmatory that the stimulation by such odorstream is olfactory. Section of the olfactory tract inhibits the reactions. Olfactory stimulation and reactions are

not affected by section of the ophthalmic branch of the trigeminal nerve.

7. Under circumstances allowing discrimination, the tadpoles of the toad prefer animal foods.

8. Such discrimination appears to rest upon the appropriate stimulation of the olfactory receptor.

9. Tadpoles of the toad show by proper reactions that animal food is recognized, although not visually perceptible.

10. The receptor organ so stimulated must be a distance receptor and thus is olfactory in function.

11. In the metamorphosed toad the visual stimulus is the principal and guiding factor in procuring food. Therefore, it is inhibitory in relation to other stimuli and their resultant reactions.

POSTSCRIPT. Since the preparation of this paper, Copeland has published in *The Journal of Animal Behavior*, vol. 3, pp. 260 to 273, an account of the olfactory reactions of the newt *Diemyctylus* and has shown that this amphibian can scent food under water as a fish does.

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THE JOURNAL

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ERRATA

"A Quantitative Determination of the Orienting Reaction of the Blowfly Larva."
THE JOURNAL OF EXPERIMENTAL ZOOLOGY, pages 213-280, volume 17, number 2.

p. 224, line 8, following "the work," insert *of these experiments*.

p. 253, line 21, for "swing" substitute *swinging*.

p. 259, line 5, insert after "marked" (*see fig 18*).

p. 267, line 12, substitute for "average" *hypothetical*.

In figure 4, E-19 was an identification mark on the original record sheet, which was reproduced by mistake and has no significance in connection with the figure. The same is the case with E-29 in figure 5; 59 in figures 7 and 8; E-19 in figure 9; 49 in figures 10 and 11; 59 in figures 12 and 13; 39 in figure 14; E-39 in figure 16, a; 29 in figure 16, b; 49 in figure 16, c, d and e; 100% 39-1st in figure 16, f; *all the numbers on figures 19 and 20.*

THE RELATIONS OF BURSARIA TO FOOD

II. DIGESTION AND RESORPTION IN THE FOOD VACUOLE, AND FURTHER ANALYSIS OF THE PROCESS OF EXTRUSION

E. J. LUND

Zoölogical Laboratory of the Johns Hopkins University

EIGHT FIGURES AND TWO PLATES

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INTRODUCTION

To obtain exact information on the processes of metabolism in single-celled organisms, and particularly the Protozoa, experimental procedure must be based upon simple, well defined and reproducible conditions, so that the work may be repeated and verified. Attempts to fulfill this requirement are subject to the

danger that the conditions may become so artificial as not to warrant conclusions concerning what occurs under usual ('normal') conditions of the organism. Nevertheless, a certain degree of simplicity may often be obtained in the conditions of experimentation and yet coincide with the usual conditions of a varied environment to a sufficient degree so that the results give a definite answer to questions which are directed toward finding out what the principles are which underlie the processes. The following experiments have been carried out with an attempt to fulfill the above requirements.

In a previous paper (Lund '14) some of the most important external relations of *Bursaria* to food have been presented, including a demonstration of a selective extrusion among the food vacuoles.

The following paper deals with the processes which take place in the food vacuole, and with the conditions under which it exists as an active system in this unicellular animal.

Proteins, fats, and carbohydrates in the form of starches are present in the material eaten by *Bursaria*, as may easily be detected by direct observation or by the aid of microchemical tests. Protein in the form of other living ciliates, flagellates, etc., is taken in and digested. Many of these Protozoa which serve as food contain fat globules and starch or amyllum grains, and hence all three food substances may at times be found present in the cytoplasm of *Bursaria* from flourishing cultures.

Since there is disagreement between results of different investigators, working with the same Infusoria; but more particularly because there are indications that similar forms among these animals differ in their food metabolism, the statements here made refer only to *Bursaria* and may or may not be valid for others, except where specifically stated.

MATERIAL AND METHODS

Simple types of the three classes of food were found in lipid-free egg yolk (vitellin), pure olein, and starch grains of various kinds. Potato starch was the most serviceable because grains of a very uniform size can be obtained by repeated decantation of a suspension, and hence results may be expressed quantitatively in terms of unit volume. Fresh hard boiled yolk—a combination, chiefly, of lipoids and protein—was also used. Olive oil of the best grade obtainable gave the same results as prepared olein.

From the yolk of the hen's egg was prepared a protein (vitellin) that was perfectly lipid-free, so far as could be determined; this was done as follows: An egg was boiled to hardness (15–20 minutes), and the granular portion of the yolk was kneaded gently to a moist, fine, floury pulp. This was placed loosely in a Soxhlet apparatus and extracted with an alcohol-ether mixture, for eight to ten hours; in this way the fat and lecithin were removed. The vitellin was then washed several times with fresh alcohol-ether mixture, removed from the Soxhlet and while still moist, gently kneaded to a fine white powder. This was left to dry at 25°C. for twenty-four hours. After drying was completed the residue was gently rubbed between sheets of filter-paper. When the steps mentioned above were carefully carried out, the grains of vitellin were found to be separated. This white dry powder was kept in a dry bottle in a cool place, and used as a food stock.

When the animals were to be fed, a suspension of the powder was made in tap or distilled water and rubbed up; then left to settle, and the supernatant liquid with the smaller particles was poured off. This process of separating the smaller grains from the larger ones was repeated until a perfectly clear suspension of the protein replicas of the uniform hard-boiled grains was obtained.

It is important to note that some eggs yield much more uniform yolk grains after boiling, than others. Only those eggs were used in this work which were satisfactory in this respect.

It seems highly probable from the results, that all traces of lecithin and fat had been removed by this method. Cold ether or cold alcohol extraction alone is not sufficient to remove all the lipid content from hard boiled yolk. This will appear from results (p. 29) of experiments in which such vitellin grains were used.

By this method of preparation, lipid-free vitellin may be obtained in very uniform grains, and this permits one to fulfill a condition most important for precise experimentation. One can feed definite unit quantities to a single cell, and this makes it possible to attack a number of questions regarding metabolism that would otherwise be inaccessible, and to express the results in quantitative terms.

Fresh hard-boiled yolk served as a protein-lipoid diet, which could be fed in the same way, for the vitellin grains are simply the protein matrix of the fresh hard boiled yolk grains in which the lipoids and any other alcohol-ether soluble substances are imbedded.

There is, of course, no means of knowing whether the vitellin of the unextracted yolk grain does not undergo change during ether-alcohol extraction; but the fact (demonstrated by the experiments) that in the food vacuole digestion of the protein in these two forms occurs in the same way and with about equal readiness, shows that the process of fat extraction does not alter the chemical or physical nature in such a way as to interfere with the digestion and resorption of the vitellin.

In all the experiments where necessary, the animals were placed in 500 cc. of tap water twenty-four hours previous to feeding. The result of this was that the food and débris contained in the cell had been discharged during the twenty-four hours of starvation, and hence a cell with perfectly clear cytoplasm was obtained for the experiments. By this means a particularly desirable uniformity in physiological condition of the organisms was obtained. The material was from the same wild stock cultures as that used in the experiments reported in my earlier paper.

In most of the experiments the organisms were fed singly, and large numbers were used, so that any individual variations play a minor part, if any, in the final results.

Where temperature regulation was necessary the experiments were carried on in an oven kept constant to within 1.5°C .

TO WHAT EXTENT CAN YOLK BE CONSIDERED A FOOD FOR BURSARIA

As yet it has not become possible to propagate pure races of *Bursaria* from single individuals, or in culture, by feeding it an artificial diet of vitellin or fresh yolk; so that it must be remembered in conjunction with the facts presented at this time, that the full requirements for the maintenance of life and reproduction have not been fully satisfied, and to this extent the ideal conditions have not been met.

But to prove that vitellin and fresh yolk are drawn upon for the energy requirements and growth of the cell Experiment I is given:

Experiment 1. Three sets of 74 individuals each were starved in tap water for 24 hours. Set A was fed with fresh hard boiled yolk in suspension prepared as described above. Set B was fed vitellin; while Set C was not fed. Each individual in the three sets was left to eat as much as it would in 20 minutes. They were then picked out and washed once in tap water which had been boiled, from this they were removed to watch glasses each containing 2 cc. of boiled tap water. The water was boiled in order to kill any bacteria, or other organisms sometimes present in small numbers in the tap water. Two individuals were placed in each watch glass and these were set in moist chambers which were kept side by side. There was therefore no difference in the temperature at which the three sets remained during the experiment, although a daily fluctuation in temperature of 4 or 5°C . occurred. Such variation, however, does not affect the results of the experiment for the particular end in view. It was found that after feeding twenty minutes nearly all of those in the fresh yolk suspension (A) had eaten large quantities, while those in the vitellin suspension (B) had on the average not eaten so large a number of grains as those in the fresh yolk suspension, although in both Set A and Set B, each individual had eaten more than one grain. All the individuals in the three sets were treated identically except in feeding. All were normal and active at the beginning of the experiment. At the end of every 24 hours detailed records were taken for each individual, as to whether it had a normal form or had undergone dedifferentiation—

which is under many conditions a typical reaction—and if the latter were the case, to what extent it had proceeded. Death was taken to have occurred when the cell had ceased to move and began to disintegrate. In this way a record of the effects of the food on the maintenance of form, degree of activity and length of life, was obtained, so that from these the means can be taken and compared.

To save space, instead of giving the detailed results in the form of tables they are given by curves and averages. Curves *A*, *B* and *C* of figure 1 represent for the three sets respectively, the longevity or death rate. Points on the abscissa indicate the time in hours, while points on the ordinate represent the number of individuals which were still alive at the time the record was taken.

The curves *A* and *B* of Sets A and B respectively, show clearly (1) that the yolk and vitellin had a definite effect in prolonging the life of the cell. The relation of curves *A* and *B* to each other will become clearer when we have considered later experiments (p. 28) which show, other things being equal, that we should expect longer life from individuals fed both lipoids and protein. By comparing the average length of life in the three sets we find that Set A lived 4.98, Set B, 5.39 and Set C 3.20 days. Sets A and B, therefore, lived on the average about two days longer than the unfed Set C. (2) The vitellin grains underwent total digestion and resorption in most cases, while the fresh yolk grains were generally only partially digested, this was especially the case where more than 2 or 3 grains were eaten. (3) The animals which had been fed were on the average more vigorous than those of Set C. (4) The animals of Set A grew to be larger in most cases than those of either Set B, or Set C. Many of Set A became twice as large as those of Set C, while those fed vitellin were on the average larger than any of Set C. The maximum size was reached about thirty-six hours after feeding.

There can therefore be no question but that both the fresh yolk and the vitellin entered into the chain of metabolic processes and were in part at least, drawn upon by the cell for its energy requirement. But very few of the organisms divided. Whether yolk or vitellin is a sufficient diet for cell division as

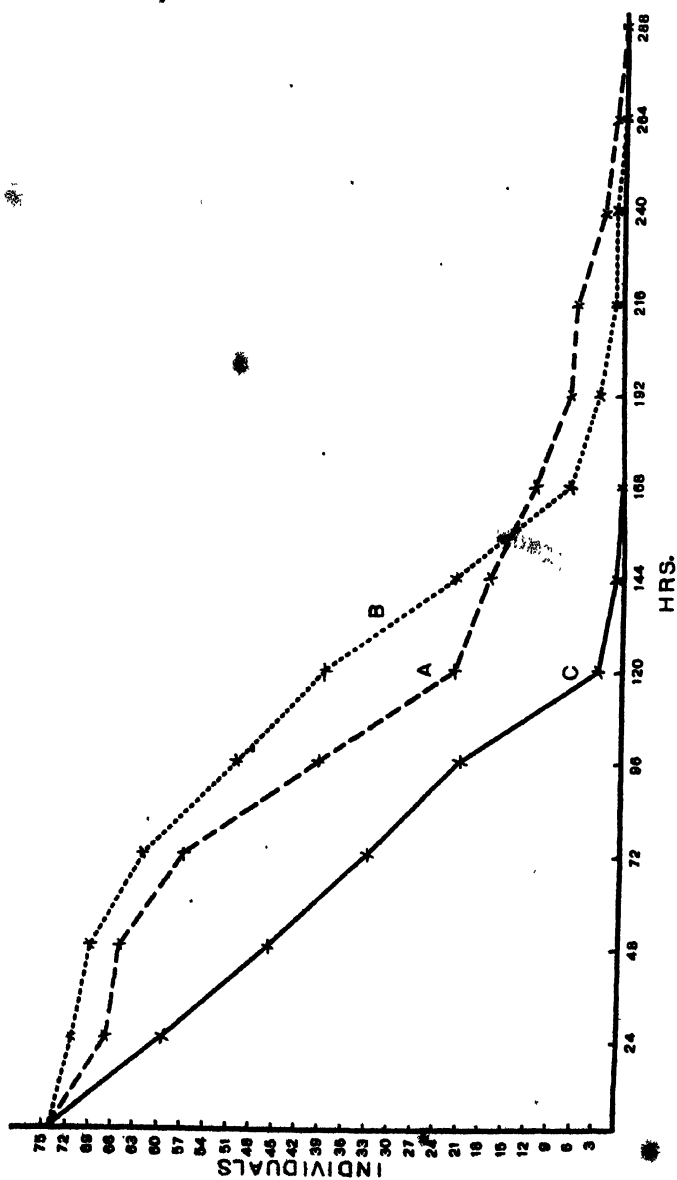


Fig. 1 Curves showing the length of life in three sets of 75 individuals each. Set A (Curve A) was fed fresh yolk; Set B (Curve B) was fed vitellin, while Set C (Curve C) was not fed.

well as for growth can not be answered at present.' But for the questions dealt with in the present paper the important fact to establish is that the yolk or vitellin can be drawn upon for the energy requirements and growth of *Bursaria*.

PROTEIN DIGESTION

1. The food vacuole; formation and physical changes

The passage of the grain into the body is brought about partially by ciliary action at the base of the buccal pouch; but large grains or masses of food are pushed through the base of the gullet and into the endoplosm, by what seem to be contractions of the wall of the base of the gullet, and also apparently by activity of the endoplasm about the gullet behind the food. During this process of swallowing, a liquid comes to be included about the food so that when the vacuole separates from the base of the gullet liquid surrounds the yolk grain.

Where does the liquid enclosed with the grain come from? Part of it is derived from the external medium, as is readily determined by direct observation of the process of swallowing. But the liquid in the vacuole, when the latter is separated from the base of the gullet, is likewise partially made up of an acid secretion from the cytoplasm of the lower portion of the gullet, as will be demonstrated below. When the vacuole has formed it is usually carried toward the middle of the cell and may remain practically stationary, especially if it is large. If movement takes place, which is nearly always the case when the vacuole is small, then the vacuole may traverse any part of the cytoplasm, and in any direction. In *Bursaria* there is no such regularity in the course of the food vacuole as has been described for *Paramecium* by Nirenstein ('05); and for *Carchesium* by Greenwood ('94). Often digestion and resorption begin and are completed in one and the same place, without any circulation of the vacuole. Residues are extruded from a small area on the dorsal side of the body.

The first visible change which takes place is the absorption of the liquid which has been enclosed with the grain during the

formation of the vacuole. This is always definite and can generally be readily observed and followed throughout the process. This is exemplified by the following experiment:

Experiment II. Single normal individuals which had been placed in tap water 24 hours previously, were each fed a single grain of fresh yolk and the time interval noted between the separation of the vacuole from the base of the gullet and the point of complete disappearance of the liquid about the yolk grain.

Table 1 shows records from ten individuals each fed one grain. From the table it will be seen that the duration of the process is relatively uniform. The point when all the liquid about the grain has been absorbed was determined to within about thirty seconds.

TABLE 1
Experiment II

INDIVIDUAL NUMBER	1	2	3	4	5	6	7	8	9	10	AVERAGE
Number of minutes for resorption of liquid about yolk grain.....	4	3½	3	5	5	4½	7	4	3½	5	4.45

When more than one grain is enclosed in the vacuole the same process of absorption takes place, following the separation of the vacuole from the base of the gullet; the vacuole membrane becoming closely applied to the surface of the grains as is shown in figure 2.

Different vacuoles in the same cell are quite independent as to the absorption of fluid. To illustrate, figure 3 is given. Here the new vacuole contained a comparatively large amount of liquid about the grain (in most cases the quantity of liquid is less), and came to be located close to an older vacuole in which the process of digestion of a *Paramecium* had practically been completed, and which contained a considerable quantity of liquid. The vacuole containing the digested *Paramecium* was unaffected in size by the absorption of the liquid about the yolk grain in the newly formed vacuole.

An explanation on the basis of osmotic relations within the cytoplasm and in the vacuoles, assuming the vacuole membrane

to remain in a uniform condition, might perhaps account for the visible difference in the vacuoles in this and similar cases. But a conception of this process may more properly be obtained if we

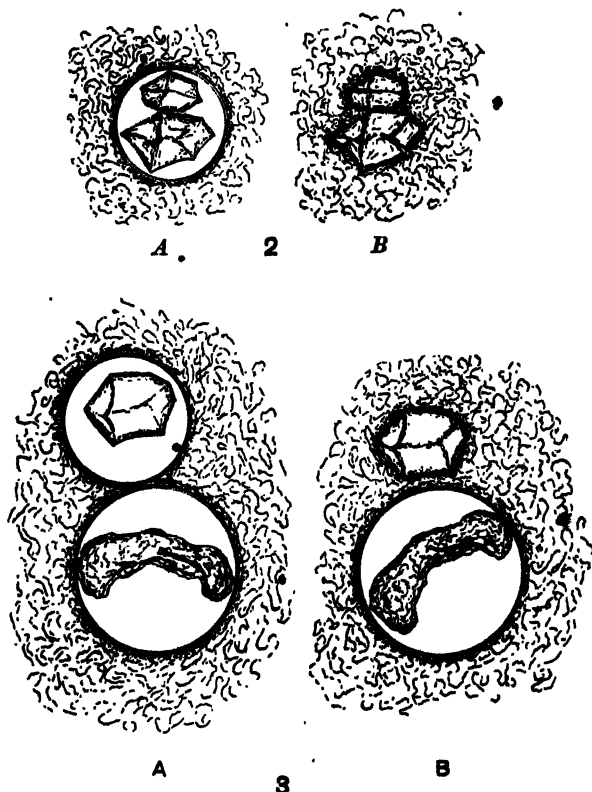


Fig. 2 Shows the resorption of the liquid included with the yolk grains during formation of the vacuole. *A*, food vacuole just separated from base of gullet; *B*, the same, five minutes after separation.

Fig. 3 Showing the independence of vacuoles with respect to resorption of liquid contents. The larger vacuole containing a partially digested *Paramecium* is not affected by the resorption of the fluid in the vacuole containing the fresh yolk grain. *B* drawn 5½ minutes after *A*.

think of it as a process of imbibition of the liquid by the colloidal cytoplasm, accompanied by changes in the permeability of the vacuole membrane. The problem here must be similar to that

which will confront us later regarding the absorption of the liquefied products of digestion.

Within one or two hours after the grain comes to lie in the vacuole, the beginning of solution becomes apparent as a liquefaction and consequent rounding off of the corners and edges. This proceeds in exactly the same manner as when cubes of coagulated egg-albumen are digested by the action of pepsin-hydrochloric acid. At the end of digestion no solid remains of the vitellin grain can be seen; all that is left is more or less liquid in the vacuole.

Another important visible change in the vacuole consists in the usual *second* appearance of liquid about the vitellin grain. It seems most in conformity with the observed facts to consider this as a consequence of the formation of soluble products of digestion, and as being due to the fact that the rate of resorption of these liquid products is less than the rate at which the liquefaction of the vitellin grain takes place.

The reason for this conclusion is partly based upon the fact that in some cases the whole process of digestion of the vitellin may go on and be completed without the appearance of any visible liquid between the grain undergoing solution, and the vacuole membrane. In this case it is obvious that the rate or power of resorption is equal to or greater than the rate of solution of the grain. Furthermore, it is to be expected that as digestion of the protein continues, the affective osmotic concentration of the cleavage products increases and this obviously may bring about an increase in the liquid contents, provided that the permeability of the vacuole membrane (resorption) does not undergo a simultaneous, proportional increase. In other words, the absence or presence of liquid during digestion of vitellin is a resultant of two sets of conditions (a) the rate of digestion and (b) the rate or power of resorption, one factor of which is the degree of permeability of the vacuole membrane. This further agrees with the fact that digestion of a vitellin grain is not always at a uniform rate. A rapid solution of the grain sometimes begins shortly after eating, changing later to a slower one. Even

food vacuoles formed at the same time, of the same material, and in the same individual, may behave diversely as to the resorption of liquid, and also in rate at which digestion takes place. But this individuality of behavior in different vacuoles does not prevent the conception of equilibrium from being applied to the phenomena of resorption. Essentially the same observable physical changes take place in vacuoles containing fresh yolk as has been described for vitellin. Further evidence for this view will be given on page 25.

2. Chemical changes in the food vacuole

Some of the chemical conditions in the food vacuole of *Bursaria* during digestion of vitellin can be shown by the use of sensitive indicators adsorbed by the grains.

Vitellin or fresh yolk grains stained with neutral red remain bright red during the whole process of digestion. Grains stained in an alkaline alcoholic solution of alizarin, quickly lose the blue color and remain colorless throughout the digestive process. Similarly, grains stained in an aqueous blue litmus solution change to red and remain red until nothing remains of the grains. These three indicators agree therefore in showing that the whole process of digestion of vitellin takes place in an acid medium, and that during no part of the process does alkalinity appear, as it does in food vacuoles of *Parmecium* and some other ciliates.

Grains of vitellin or yolk stained with congo red become dark brown after one, two or more hours, and continue to remain dark brown throughout digestion. In some grains no change in color from red to brown takes place, but what differences in conditions account for this has not been determined. Two other indicators were used, Tropaeolin 00 and diethylaminoazobenzene. Vitellin or fresh yolk stained with Tropaeolin 00 showed little or no change in color. Diethylaminoazobenzene likewise showed no change. This result is due to the fact that these two indicators are not sensitive to very weak acids or strong acids in high dilution.

The points of origin of acid as well as the rate of acidification of the vacuole contents was determined. Table 2 gives typical records of the total time for completely acidifying grains in fifteen out of fifty individuals which were fed with fresh yolk stained in blue litmus. No noticeable difference existed between the acidification of fresh yolk and that of vitellin. The time in seconds which it took to change completely the blue litmus-stained grain to red, is given by the numbers underneath the number of the individual. The average of the observations in table 2 is $38 \pm$ seconds.

TABLE 2

Total time in seconds for acidifying fresh yolk grains stained in blue litmus. The table shows typical records of fifteen out of fifty individuals in which the time for acidification of each grain was taken. The grains which are represented by the numbers opposite to the brackets were included in the same vacuole. The other numbers, not opposite brackets, represent grains, each of which were in a separate vacuole, i.e., swallowed separately

	INDIVIDUAL NUMBER														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1st grain.....	15	20	{ 10	25	20	65	45	60	80	45	15	{ 30	{ 45	{ 35	55
2d grain.....	35		{ 30	20	15	40	40	10	30	20	30	{ 30	{ 60	{ 35	30
3d grain.....	50		{ 55		15	80		50	20	30		{ 40	{ 50	{ 35	25
4th grain.....			40		15				25	60		45	{ 40	{ 35	
5th grain.....			80		20								60	{ 30	
6th grain.....			30		30									{ 45	
7th grain.....					20									{ 75	
8th grain.....					180									{ 30	

It was evident that the time of acidification depended mainly if not solely upon three conditions, as follows: (a) the size of the grain; the larger the grain, other things being equal, the longer it took for the last trace of blue color to disappear. For example; grain number 8 of individual number 5, table 2, was a very large grain; while grain 2 of individual number 8 was very small, hence the difference in time. (b) The physical consistency of the grain must be assumed to determine its permeability and hence its time of acidification. (c) The concentration of the acid secreted into the vacuole.

It has so far not been possible to determine the relation between the number of grains eaten and the time of acidification of each, because litmus is slightly toxic to *Bursaria* and hence stained grains of even fresh yolk are not eaten very readily.

The arrow at *a* in figure 4 shows the point at which the acid first begins to be secreted into the forming vacuole. This is indicated by the sudden appearance of a faint pink color about the edge of the grain which gradually increases. Figure A in plate 1 shows the degree of acidity reached before the grain leaves the gullet.

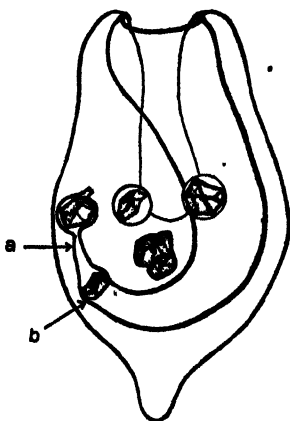


Fig. 4 Outline drawing of *Bursaria* showing steps in the formation of food vacuoles and resorption of the liquid enclosed; *a*, place at which acid secretion begins. Grains of relative size shown will not as a rule be rejected after they have passed the point indicated by *b*.

As the grain passes through the mouth surrounded by the mixture of the acid and the external medium, the change toward red progresses rapidly until the grain's interior has been reached by the acid (plate 1, figs. A, B and C): When the acid had reached the center of the grain the times noted in table 2 were taken. There is no way of telling whether acid is continuously poured into the vacuole after it has left the end of the gullet. No evidence was obtained as to the nature of the acid secreted, nor as to whether it is in combination or in the free condition.

The physical and chemical changes taking place in the vacuole containing vitellin or fresh yolk grains, which have been described above, are briefly summarized in plate 1. Here the series of figures from A to L, inclusive (series 1 and 2), show the usual course of the process of digestion and resorption of fresh yolk when the latter is retained throughout the process. Series 1 and 3 show the usual course of digestion and resorption of vitellin, when the rate of resorption is less than or equal to the rate of digestion. Series 1 and 4 show the same when the rate or power of resorption is equal to or greater than the rate of digestion of the vitellin grain. The successive figures do not represent the condition at equal intervals of time, but are typical stages in the process. Frequently there occurs an alternate presence and absence of liquid or smaller variations in the quantity of liquid around the grain during the stages from H to L, inclusive, and from H' or H'' to L' or L'', inclusive. Thus the figures from H, H' and H'' to L, L' and L'' respectively, represent this process only as it proceeds typically in the majority of cases.

3. Effect of quantity of vitellin eaten upon the average velocity of digestion

Since each cell could be fed just the desired number of grains of practically uniform size, the effect of the quantity of protein upon the average rate of digestion, and upon certain other processes could be determined.

Experiments arranged to find out what the relation is between rate of digestion and the quantity eaten, were tried with both fresh yolk and vitellin. Fresh yolk was found unsatisfactory for this purpose, since the grains were generally not retained but extruded before digestion was completed. This was especially true when a large quantity of fresh yolk had been eaten (cf. Experiment VIII, p. 34). Under usual conditions vitellin fed to the animals was not extruded, and this was especially true if care was taken not to let the animals eat too many grains. The average limit in the number of grains that could be eaten and retained was determined from a large number of observations

during the work. In these it was found that when normal, active animals were fed vitellin under what seemed to be the best experimental conditions, five to seven or even nine grains were retained, and digestion of the whole mass continued to completion, leaving no residues.

In the experiments, to determine the average rate of digestion of vitellin the maximum number of grains fed was six, and it was found that with this number as the maximum extrusion very rarely took place.

Rise in temperature accelerates the digestive process, but the variations due to temperature were eliminated, since all the sets of individuals were side by side in an oven kept at 25 to 26°C., and the fluctuations in temperature due to taking the moist chambers from the oven while the records were taken, were the same for each set of individuals.

The organisms used in each experiment were from the same healthy culture. They were starved in tap water twenty-four hours previous to the beginning of the experiments, so that a clear cell was obtained.

When the animals were to be fed, a large number were removed from the 500 cc. dish of tap water in which they had been starved, to an 8 cc. dish containing tap water. Some of the vitellin suspension prepared as described above (p. 3), was added to the tap water in the dish containing the animals. The quantity of vitellin suspension added varied according to whether it was desired to feed a small or large number of grains to each individual. If a large number of individuals were to be fed only one grain, a very weak suspension was added; the result was that the rate of feeding was slow, and hence gave sufficient time to remove the individuals as soon as one grain had been swallowed. As feeding went on, the individuals that had eaten the desired number of grains were picked out with a pipette and placed in separate dishes containing tap water. In this way, under favorable conditions, it was possible to obtain several sets of individuals simultaneously, each individual of each set having eaten a definite number of protein grains. After feeding, the animals were

removed from the dishes with tap water, and two individuals were placed in each watch glass in order to lessen the labor in taking the records. Each watch glass contained 2 cc. of tap water. The watch glasses were placed in moist chambers. In order to avoid the effect of individual variations in the rate of digestion of equal numbers of grains, forty-eight individuals were used in each set. Effects upon the result due to variation in size of the grains were therefore also practically avoided. Digestion was taken to be complete when all the solid contents of the vacuole had become luquefied. The results of two experiments are given.

Experiment III, February 12, 1913: Table 3. Each individual of Set A, Set B and Set C was fed 1, 3 and 6 grains respectively. Examination of each individual was made and records taken, $3\frac{1}{2}$, 5, $6\frac{1}{2}$, 8, $9\frac{1}{2}$, 11, $12\frac{1}{2}$, 14 and 22 hours from the time of feeding.

The rate of digestion and resorption when a large number of grains have been fed is in many cases slow toward the end of the process; so that it is sometimes difficult to tell at just what time (within 1 or $1\frac{1}{2}$ hours) the last remains of solid protein disappear. For this reason eighteen hours was taken as the average time of complete digestion in those individuals of Set C which had small protein residues at the end of fourteen hours and none at twenty-two hours. All of Set C showed complete digestion at the end of twenty-two hours.

In each of the three sets (table 3) individuals numbered 2 show a longer time for digestion than their partners numbered 1. This has no significance, because it is due to arbitrarily calling the first individual that had completed digestion, number 1, and the last one, number 2; when the record was taken. The same is true for table 4, Experiment IV.

It will be seen from table 3 that the average number of hours which it takes to complete digestion in the three sets A, B and C, is not directly proportional to the number of grains eaten, i.e., as 1:3:6. Before further consideration of these results the next experiment will be described.

TABLE 3
Experiment III: February 12, 1913—Number of hours for complete digestion
 Set A—Forty-eight individuals, each fed 1 grain of vitellin

	WATCH GLASS NO.																								TOTAL NUMBER HOURS TO COMPLETE DIGESTION	AVERAGE NUMBER HOURS TO COMPLETE DIGESTION
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		
Individual No. 1.....	5	5	6½	6½	8	6½	6½	5	6½	5	8	6½	6½	5	5	6½	5	5	6½	6½	5	5	5	6½	339	7.06
Individual No. 2.....	9½	8	6½	9½	8	9½	12½	9½	6½	9½	8	8	8	8	6½	8	6½	8	6½	9½	6½	9½	5	6½		
Set B—Forty-eight individuals, each fed 3 grains of vitellin																										
Individual No. 1.....	8	8	5	9½	6½	5	6½	9½	14	8	6½	9½	6½	11	6½	5	6½	11	6½	11	6½	11	9½	5	439.5	9.15+
Individual No. 2.....	9½	11	9½	9½	14	9½	8	12½	14	9½	9½	8	12½	8	8	8	14	8	14	14	14	11	11	5		
Set C—Forty-eight individuals, each fed 6 grains of vitellin																										
Individual No. 1.....	18	18	9½	9½	18	9½	14	11	14	14	11	12½	12½	11	14	14	12½	18	12½	14	18	9½	18	18	726	15.12+
Individual No. 2.....	18	18	12½	11	18	18	18	18	18	18	18	18	12½	12½	18	18	14	12½	18	14	18	18	12½	18		

Experiment IV: Table 4. In this the procedure was the same as in the previous experiment, but the animals were taken from a different culture, and instead of 3 sets of 48 individuals each, 4 sets of 48 individuals each were used. Each individual of Sets A, B, C and D were fed 1, 2, 3 and 6 grains, respectively.

Four individuals (marked X in the table) of Set C extruded part of the six grains and are therefore not counted in the results. The temperature was 25 to 26°C. Examinations were made 3½, 5, 6½, 8, 9½, 11, 12½, 14, 15½, 17, 18½, 20 and 22½ hours after feeding. In this experiment the possible slight error in the average time for complete digestion in Set C, Experiment III, due to the approximation of the time of complete digestion in some of these individuals, is eliminated, because observations were made regularly throughout the whole 22½ hours. And the conditions were on the whole better than in Experiment III.

The results of both experiments agree in showing that the average total amount of vitellin digested per unit of time is greater in individuals fed a larger quantity than in those fed a smaller one, i.e., this quantity is greatest in Set D > Set C > Set B > Set A.

The average time, found by experiment, for the complete digestion of one grain or its equivalent in volume or mass, in the sets of individuals of the preceding two experiments is given in column 2 of table 5.

Now if we establish a limiting case, by supposing that the average quantity of vitellin digested per unit of time, is a function only of the amount of surface of the substrate exposed to the action of the digestive agent, then we should expect to find (provided further that the total surface of each grain in the individuals fed more than one grain was exposed to the action of the digestive agent), that individuals fed 6, 3 and 2 grains each, would complete digestion of all in the same length of time as it takes to digest one grain. Hence, if it takes 8.56 hours (Experiment IV) to digest one grain, then in Sets B, C and D we should expect to find that the quantity of vitellin equal to the volume of 1 grain, would undergo digestion in the times shown in table 5, column 3.

The results are uniformly *less* than those found by experiment (column 2).

Now since we have constructed a limiting case for the maximum average rate of digestion on the assumption that the rate is in direct proportion to the surface of substrate exposed, we may on the same assumption establish a limiting case for the minimum average rate of digestion, by assuming that the surfaces exposed to digestive action in the four sets, are to each other as the surfaces of spheres of vitellin having volumes 1, 2, 3 and 6. Calculating this on the basis of the rate of digestion found by experiment for one grain (Set A), we obtain the values given in column 4, table 5. It will be noted that these values, based on the above assumption are all *greater* than the values found by experiment. The results of both experiments agree. However, the intermediate position of the obtained results (column 2, table 5) does not exclude the possibility that the digestive process

TABLE 5

Summary of and calculations for the average velocity of digestion of vitellin, based on results of Experiments III and IV, tables 3 and 4

EXPERIMENT	SET	COLUMN					Value of t in $t = k\sqrt{M}$
		1 Number grains fed to each individual	2 Average time found for complete digestion of one grain, or its equiv- alent	3 Calculated average time for complete digestion of one grain or its equiv- alent, on basis of sur- face, grains separate	4 Calculated average time for complete digestion of one grain or its equiv- alent, on basis of sur- face, total number of grains a sphere	5 Average time for complete digestion of one grain or its equivalent, calcu- lated from $t = k/\bar{M}$	
			hours	hours	hours	hours	
III. Febru- ary 12, 1913	Set A...	1	7.06				7.06
	Set B...	3	3.05	2.35	4.89	4.06	5.28(?)
	Set C...	6	2.52	1.17	3.88	2.846	6.17
IV. Febru- ary 18, 1913	Set A...	1	8.56				8.56
	Set B...	2	5.62	4.28	6.76	6.04	7.96
	Set C...	3	4.25	2.85	5.93	4.94	7.38
	Set D...	6	3.04	1.42	4.70	3.49	7.44

really conforms to the law of a heterogeneous chemical reaction (between a solid and a liquid).¹

The values in column 5, table 5, were obtained by substitution in Arrhenius' formula $t = k\sqrt{M}$ for the rate of digestion in the dog as found by London.² The values obtained by means of this formula agree well, within the limits of experimental error, with those found by experiment in the case of *Bursaria*.

In column 6, table 5, are given the values of k in the formula $t = k\sqrt{M}$. The agreement is not so good in Experiment III as in Experiment IV for reasons given above, although the variation in the constant k of both experiments, and especially that of Experiment IV, is well within the limits of experimental error.

If it were possible, it would be interesting to determine what the value of k would be for other food substances.

The experiments show that in spite of individual variations among the organisms and the variation in the process in different vacuoles in the same individual which were mentioned above, the sum total gives a definite result, and shows that the average rate of digestion of vitellin under rigid experimental conditions follows a definite law.

4. *Effect of congo red upon the average velocity of digestion, extrusion, etc.*

In order to discover some of the changes which take place in the reactions of the cell when protein (vitellin grains) was changed by letting it adsorb a substance from solution, the following experiment was carried out. Congo red was chosen since its toxicity to *Bursaria* is low when compared to that of many dyes,

¹ The intermediate position of the values found suggests agreement with the results of the experiments of Bayliss ('04), who found that "with concentrations of casein up to about 4 per cent, the velocity of digestion is proportional to the concentration of the substrate . . . whilst with more than 8 per cent, inverse proportionality sets in" (cited from Euler: General chemistry of the Enzymes, p. 186). Furthermore the results also suggest the possibility that the quantity of active agent produced in the cell or vacuole is not directly proportional to the quantity of vitellin eaten.

² This is, $t = k\sqrt{M}$; where t is the time for complete digestion, M the amount by weight eaten, and k a constant depending upon the nature of the food.

and since vitellin grains readily adsorb it in considerable quantity, depending upon the length of time during which the grains are left in the aqueous solution of the dye.

The results of the experiment clearly show: (a) that the adsorbed congo red very markedly interferes with or prevents digestion of the parts of the vitellin grain to which the congo red has been adsorbed, and (b) that it brings about a condition which leads sooner or later to an extrusion of the contents of the vacuole; (c) that it may exert a greater or less toxic effect from within the vacuole, upon the cell, which may lead to an earlier death than if the unstained vitellin had been eaten. In short: *the chemical nature of the substance taken into the vacuole of Bursaria may determine in various ways what many of the conditions and reactions of these organisms will be*, and especially, in this connection, the action of the digestive agent and the process of extrusion.³ The following are the results in brief.

Experiment VI. Grains of vitellin of uniform size, prepared as described above, were stained twenty minutes in a deep red aqueous solution of congo red. They were then washed several times until no more stain could be washed out. An unstained portion of the same vitellin sample was washed the same number of times in tap water; this was fed to the control individuals. Forty normal individuals previously starved in tap water for 24 hours were each fed one grain of the stained vitellin. Similarly a control set of 40 individuals were fed, each one grain of the unstained vitellin. All the conditions and material were the same in the two sets, except that the individuals of one set was fed stained grains, those of the other set unstained ones. Both sets of individuals were kept in watch glasses, each containing 4 cc. of spring water. Two individuals were placed in each watch glass. Records of each individual were taken 2, 4, 6, 7, 9, 11, 13 and 22 hours after feeding.

Table 6 gives the results. Table 6 (a) shows that the partially digested or undigested congo red stained grains were to a large extent extruded, while in the control set, all retained the unstained

³ I have here used the term 'chemical nature,' and in my previous paper (Lund '14) such terms as 'toxicity' (p. 29), 'specific chemical properties' (p. 41), with a general meaning. The finer distinction between the effects due to chemical and physical properties of a substance, as for example its solubility in the plasma membranes, state of colloidal aggregation, chemical reaction with the protoplasm, etc., remains an open question.

protein grains. This, therefore, shows that the *chemical* nature of the substance in the vacuole may, and does in this case, determine whether it shall be extruded or not. Table 6 (b) shows the effect of the adsorbed dye upon the process of digestion of vitellin. The stain retards and sometimes even prevents digestive action. This was seen from the fact that the corners and edges of the grains where little or no unstained yolk exists, persist more markedly than those of the unstained grains. The stained grains, nevertheless, often became smaller by shrinkage, caused apparently by solution of the protein from the deeper parts of the vitellin grain, which may have been stained less deeply. That the adsorbed dye interferes, to some extent, with the maintenance of the normal form and outline of the animal is shown by the results of table 6 (c). The fact that, for example, the total number of normal individuals of those fed a stained grain has increased rather than decreased at the eleven-hour record, since the nine-hour record was taken, is due to regulation of form, either by closing and opening the oral pouch, or by undergoing

TABLE 6

Showing the results of feeding one grain of vitellin stained in congo red, to each of a set of forty individuals. For comparison (control) another set of forty individuals were each fed 1 grain of unstained vitellin

		TIME IN HOURS AFTER FEEDING								
		2	4	6	7	9	11	13	22	
(a)	Total number of individuals that had extruded grains before death	Stained.....	0	0	1	4	9	18	25	27
		Unstained.....	0	0	0	0	0	0	0	0
(b)	Total number of individuals that had completed digestion of grains	Stained.....	0	0	0	0	0	0	0	0
		Unstained.....	0	0	1	4	10	22	28	37
(c)	Total number of individuals that were normal in form	Stained.....	38	35	27	26	15	24	23	14
		Unstained.....	37	38	37	38	34	33	35	29
(d)	Total number of individuals dead	Stained.....	0	0	0	0	0	3	4	11
		Unstained.....	0	0	0	0	0	0	0	2
(e)	Total number of individuals with a perceptible amount of liquid in vacuole	Stained.....	0	0	0	0	3	2	0	0
		Unstained.....	0	4	7	13	13	16	3	2

more fundamental morphological changes. A full consideration of these processes will be given at a later time.

That the presence of the dye also brings about a tendency to an earlier death of the individual is indicated by table 6 (d). The record for this is not complete because when the experiment was stopped a large number of the individuals still remained alive, as the table indicates.

It was stated above (p. 11; cf. figs. H, H' and H'' to L, L' and L'' respectively, plate 1) that the rate of resorption during digestion of vitellin may be equal to, or less than, the rate of solution of the grain. This is a definite and observable condition, for in some cases solution of the grain may go on with the vacuole membrane closely applied to the grain, while in other instances, even in a sister vacuole in the same cell, the process of dissolution of the grain may go on while the latter is floating in the liquid in the vacuole, which is made up, in part at least, of the liquid products of digestion. The quantity of liquid present in such vacuoles during digestion may vary, and when only a smaller quantity is present around the grain, it becomes more difficult to observe its presence; but, when the quantity is sufficient to be seen, then records may readily be taken giving (practically) the absence, or presence of liquid about the grain. The results from Experiment VI are given in table 6 (e).

It is evident that there was a much greater tendency toward accumulation of liquid about the unstained grain than in the vacuole having the stained grain. In other words, *along with decrease in digestive action upon the stained grains went a strong tendency toward absence of liquid in the vacuole.* We have therefore in this result further good evidence for the essential correctness of the statement made above (p. 11) that the rate of digestion of the vitellin may be (1) greater, (2) equal to, or (3) less than the rate or power of resorption.

Further evidence for this is shown in table 6 (e), for *during the height of digestive activity, which in this case occurred between six and eleven hours, the tendency toward presence of liquid in the vacuole is greatest.*

It is not to be supposed that the appearance of liquid in food vacuoles generally, under other conditions, need necessarily be due to rapid digestion and slow resorption (cf. fig. 3). In such cases we may have other factors bringing about accumulation of liquid.

Other tests of the effect of congo red upon digestion gave similar results, but since records of individuals were not taken these results are not expressible quantitatively.

DIGESTION AND RESORPTION OF FAT

1. *A demonstration of fat digestion and resorption in the food vacuole of Bursaria*

Nirenstein ('10) and Issel have furnished a simple and reliable method for determining the presence of lipoids in infusoria.⁴ The method consists simply in placing a small drop of the culture fluid containing the Infusoria on a slide and killing and staining by addition of a few drops of an alcoholic solution of Sudan III. The mount is cleared with NH_4OH followed by thick glycerine. By proper manipulation beautiful mounts may be obtained. The gross distribution of lipoid in the cell is faithfully preserved by this method. This is shown by the fact that fat globules of the same typical sizes as in the mounts may readily be distinguished in the ground substance of the living protoplasm and followed during protoplasmic movement especially in individuals with high fat content. Bursariae taken directly from healthy cultures always had more or less fat present in the protoplasm. This fat is probably derived from other Protozoa which serve as food.

When an individual from a wild culture, with a high fat content, is isolated and placed in tap water, the protoplasm usually becomes clearer after one or more days; showing that fat, as fat droplets at least, disappears from the cell.

The chief reason for the difference in results and conclusions of Nirenstein ('10) and Staniewicz ('10), as to whether Infusoria,

⁴ The words 'lipoid' and 'fat' are used here to designate mainly those fat-like substances in the cell which are amenable to detection by microchemical tests.

particularly *Paramecium*, actually do digest, resorb, and utilize fat for the energy requirements of the cell, appears to be the fact that these investigators did not compare the fat contents of the *same* cells nor *sister* cells, before and after feeding of fat, but limited their comparison to different individuals taken at random from the same culture.

In order to leave absolutely no question as to the fact of digestion and resorption of fat in *Bursaria*, cells which were in the process of division were isolated from the wild cultures. * Those which had many vacuoles were discarded, and in most cases only those with no food vacuoles were used. Each pair was placed in a separate watch glass with tap water. After the completion of division, or after eighteen to twenty-four hours' starvation in the tap water, one of the sister cells was fed an emulsion of olein or pure olive oil (both gave the same results), while the other sister cell was kept as a control in a separate watch glass with tap water, under exactly the same conditions.

In different experiments, at different intervals after feeding, both sister cells were tested simultaneously for the amount and distribution of the fat content. In every experiment, after a sufficient length of time, the cell which had eaten and retained the olive oil or olein for some time showed an abundance of fat droplets distributed throughout the cytoplasm, while the remaining fat in the vacuoles appeared as large, single fat droplets or as groups of such.

The fat content of food vacuoles can of course be readily seen and followed in the living cells. In the control (sister) cell a much smaller quantity of fat was invariably present, this quantity depending upon the original amount present in the cell before isolation.

That the fat is distributed equally to sister cells in dividing individuals which have no food vacuoles at the time of isolation, was of course tested by mounting sister cells and comparing the fat contents.

The result of a typical experiment is given by the camera lucida drawings. Plate 2, figure A, shows the control with a small fat content twenty-four hours after the time when its sister

cell (fig. B) had been fed olive oil. Figure B shows the sister cell twenty-four hours after feeding with olive oil. The large fat drop in B constituted the contents of the food vacuole previous to mounting. Both cells were starved in tap water for eighteen hours previous to feeding of sister cell (fig. B). The same results were obtained when fresh yolk grains were fed.

These experiments prove conclusively that in *Bursaria* the fat in the food vacuoles passes out in one form or other through the membrane of the food vacuole, and becomes distributed in the cytoplasm as small droplets. This agrees perfectly with the results obtained by Nirenstein ('10) for *Paramecium*. Liquid may at times be present about the fat droplets undergoing resorption in the vacuole, just as in the case of vitellin or yolk (plate 1, H to L). But no attempt was made to study its changes. Results from a study of the chemical changes involved in digestion and resorption of fat will be given elsewhere.

2. Rôle of fat in growth and energy requirement

The tail, in the form of *Bursaria* studied, serves in general, as a convenient index of the degree of 'fatness' or 'leanness' of this organism.

When olive oil is fed to a large number of *Bursariae* under favorable experimental conditions, so that more or less resorption takes place, the fed animals, on the average, become larger than the unfed. The tail on the average also assumes more pronounced outlines than those of the control. In such large individuals when in sufficient number for comparison with the controls, there is indication also that the length of life is on the average increased by having eaten olive oil. When individuals fed a definite number of grains (1 or 2) of fresh yolk, are compared with others fed the same number of fat-free yolk grains, the individuals fed with fresh yolk, become on the average larger, many of them attaining a relatively enormous size. During this growth perfect proportions of form are maintained. Such large individuals are always found to contain much fat. The part of the Curve A

(fig. 1, Experiment I, from 144 to 288 hours, represents individuals of this kind and constitutes evidence that the length of life of individuals fed fresh yolk is greater than that of those fed fat free yolk. During this time the quantity of fat in the cell decreases as starvation continues, indicating that the fat is used up.

Under some conditions no traces of fat can be found in Bursaria by the use of Sudan III, but this of course does not prove its complete absence. The apparent absence of fat is rare and it was found difficult by means of starvation to remove the last traces.

3. Is fat formed from vitellin or starch in Bursaria?

When yolk is extracted repeatedly with cold ether or with cold alcohol, at intervals, for twenty-four hours or more, a white residue remains, which is chiefly vitellin. Such vitellin was fed to Bursariae which had been starved. An abundant fat deposit appeared in the cytoplasm, indicating that yolk extracted with cold ether or alcohol alone still contained a fraction which appeared as fat in the cells. This was tested by extracting fresh hard boiled yolk with ether-alcohol mixture for ten to twelve hours in a Soxhlet apparatus. Vitellin so extracted was fed to one of each pair of sister cells while the other cell was kept as control. No difference appeared in the amount of fat present in the two cells, although many attempts to demonstrate a difference were made.

In view of the fact that crucial control tests were not made by Nirenstein ('10) the slight difference in fat content given in his figures 7 and 1, plate 1, cannot I think, be considered proof that the fat found to be present in Paramecium (fig. 7) after digestion of egg albumen, had been derived from the latter; furthermore, analyses of egg albumen show traces of the presence of lipoids, such as fat, cholesterin and lecithin.

Various kinds of starch and paramylum grains which were retained by Bursaria, remained intact for days and showed no signs of corrosion or solution.

THE NATURE OF SOME OF THE FACTORS WHICH BRING ABOUT
EXTRUSION*1. Experiments with paraffin oil and olive oil*

If a conception can be obtained of what some of the dynamic conditions of the cell are, by a study of the processes of intake of foods and other substances, then we might expect to find that the process of extrusion will serve as a valuable index in discovering what some of the responses of the cell system are to those same substances, after they have produced their effects from the interior of the cell. Relations as interesting as those discovered in feeding ought to be found by studying the phenomena of extrusion, though the process, for various reasons, does not lend itself so readily to experimental inquiry.

I have shown (Lund '14) that when *Bursaria* has been fed Sudan III or Chinese ink alone, these substances are not retained within the cell for any considerable length of time, and further that if *Bursaria* is fed at the same time one of these substances and vitellin, then the Sudan III or Chinese ink is extruded after a short time, provided it has not come to be located in the same vacuoles which contain vitellin; while the vitellin is retained, digested and resorbed. In other words there exists a selective extrusion among food vacuoles formed at the same time, or different times. Above (p. 24, table 6, a) it was further shown that in the case of vitellin grains stained with congo red, a large number were extruded during digestion, or before any digestion had taken place, while unstained grains were retained and digested by the control individuals. Here the condition which brought about extrusion can be only the presence of the absorbed dye, i.e., a chemical condition. Since both sets of individuals were fed one grain each, the size or shape of the grain had nothing to do with the difference between the results.

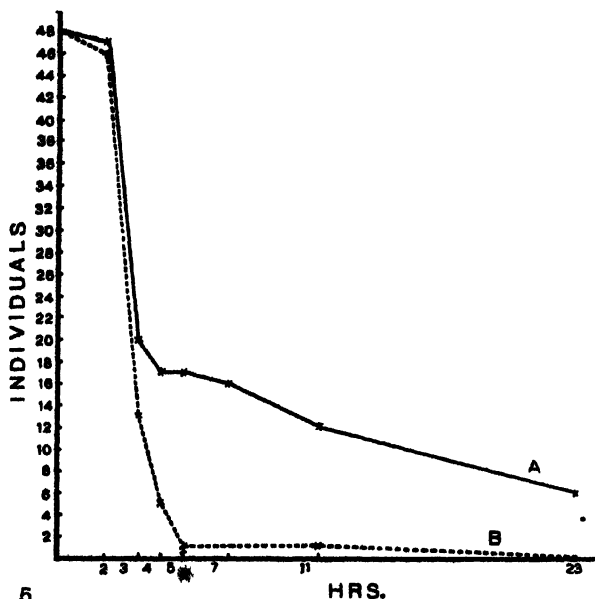
When both unstained grains, and grains stained with congo red, were fed to the same individuals, similar results were obtained. Further to test this by feeding two liquid substances somewhat similar physical properties, but of different chemical nature, the following experiment was carried out.

Experiment VII, a, b and c: (figs. 5, 6 and 7). Emulsions of paraffin oil, and olive oil were made in tap water. The animals were starved as usual in tap water. 2 cc. of this tap water was placed in each watch glass and used as a medium, so as to prevent any effect due to transfer from this into fresh tap water. Set A consisting of 48 individuals that had eaten olive oil were picked out from the 8 cc. with olive oil emulsion in which they had been feeding for 10 minutes. Another set, B, of 48 individuals that had been fed an emulsion of paraffin oil were picked out at the same time. Both sets of individuals were washed in some of the tap water in which they had been starved. After washing, two individuals were placed in each watch glass, the latter being kept in moist chambers. The moist chambers were placed in a constant temperature oven kept at 25 to 26°C.—an unnecessary precaution for the purpose of the experiment. Records were taken at the time intervals given by the points on abscissae in the curves.

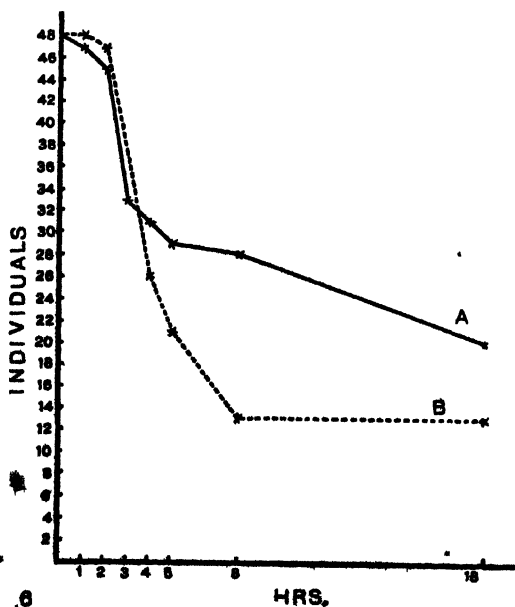
The process of extrusion was gradual, i.e., all the paraffin oil present in an individual was not usually extruded at the same time. Small globules or traces generally remained for various lengths of time after the main bulk of the oil had been extruded. Since there was no way of expressing the course of this process quantitatively, when oil was used, the time when all trace of oil in the food vacuoles had disappeared was taken as the time of extrusion, for comparison in the two sets of individuals, A and B. Hence the curves are based on the time for complete extrusion. As a matter of fact, the difference in the time of extrusion between Set A and Set B was actually greater than the curves show, for the bulk of the paraffin oil was extruded, on the average, considerably earlier than that of the olive oil.

The experiment was carried out three times, all giving concordant results, as shown by curves, figures 5, 6 and 7. In no individual in the above experiments was the disappearance of olive oil due to retention and complete digestion. Fats are resorbed very slowly. The material came from three different wild cultures, otherwise the procedure was the same in all. The extrusion reaction was apparently not as effective in Experiment VII, *b* (fig. 6) as in the others.

Tests for fat content showed marked resorption in those individuals which had retained some or all of the olive oil for some time after feeding. No increase in the fat (oil) content could be seen to have taken place in individuals of Sets B fed paraffin oil.

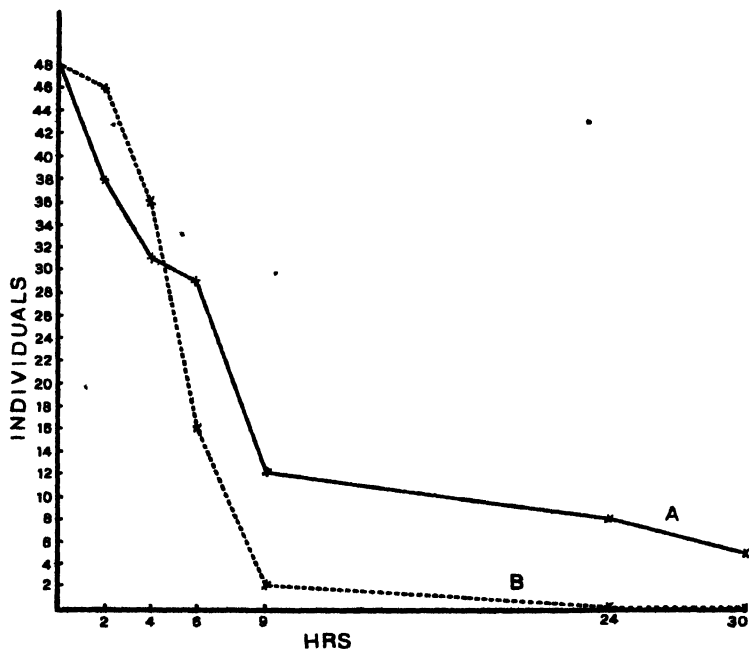


5



6

Figs. 5, 6 and 7 Curves of three separate experiments (VII, a, b and c) showing the course of complete extrusion among individuals fed olive oil (Curves A), and paraffin oil (Curves B). Points on the ordinates indicate the total number of individuals still containing oil at the time indicated on the abscissae.



7

The Sudan III method serves for the detection of paraffin oil as well, since this stains red also.

These experiments with oils again show that the retention or extrusion depends, at least in part, upon the chemical nature of the substance, and that the food vacuole functioning as an organ of extrusion may vary in its response to qualitatively different chemical stimuli.

Does the extrusion of a substance depend *alone* upon a response to the quality of a chemical stimulus by the vacuole and whatever other part of the cell system it may be specially related to in this response? If there are other factors concerned, what are they?

2. Effect of mass or volume upon the extrusion reaction

From previous observations it had become evident that extrusion depended in some way upon the quantity eaten, and accordingly the following experiment was carried out:

Experiment VIII (table 7, fig. 8). Three sets, A, B and C, of 48 individuals each, were fed fresh yolk. Individuals of Set A were fed 1 grain each, those of Set B, 3 grains each, while those of Set C were permitted to eat a considerable number of grains, at least 5 or more. Several of Set C ate 12 to 15 grains. The number of grains present in each individual of Set C was counted when the first record was taken, 3 hours after feeding. Several individuals of Set C had by this time extruded a number of the grains eaten three hours previously.

The count of the number of grains present in each individual of Set C was not made immediately after feeding, because it becomes increasingly difficult to count the number of grains in active individuals that have eaten more than 7 or 8 grains. The average number of grains in each individual of Set C, three hours after feeding, was 5.64. The number of grains in each varied from 4 to 7. The total number in the 48 individuals Set C, at the end of three hours was 271 grains.

Records of the number of grains in each individual of the three sets were taken 3, 5, 7, 9, 12, 14, 16, 24 and 30 hours after feeding. The results are given in brief in table 7, and by the curves in figure 8. The Curves A, B and C show the relative rates of total loss of the fresh yolk by Sets A, B and C, respectively.

The table shows that the total number of grains present in all the individuals of each set, decreases with increase in the length of time after feeding. The rate of decrease in these numbers is on the average in the order: Set C > Set B > Set A. This becomes more apparent if we calculate the percentage of the total number of grains fed which was present in the individuals at the times when the records were taken. In order to bring out these relations more clearly the curves in figure 8 were plotted from the percentages given in table 7.

Now, from what has thus far been said, and from the records, we have no proof that the loss in the number of grains which did

TABLE 7

100

[illegible][illegible]

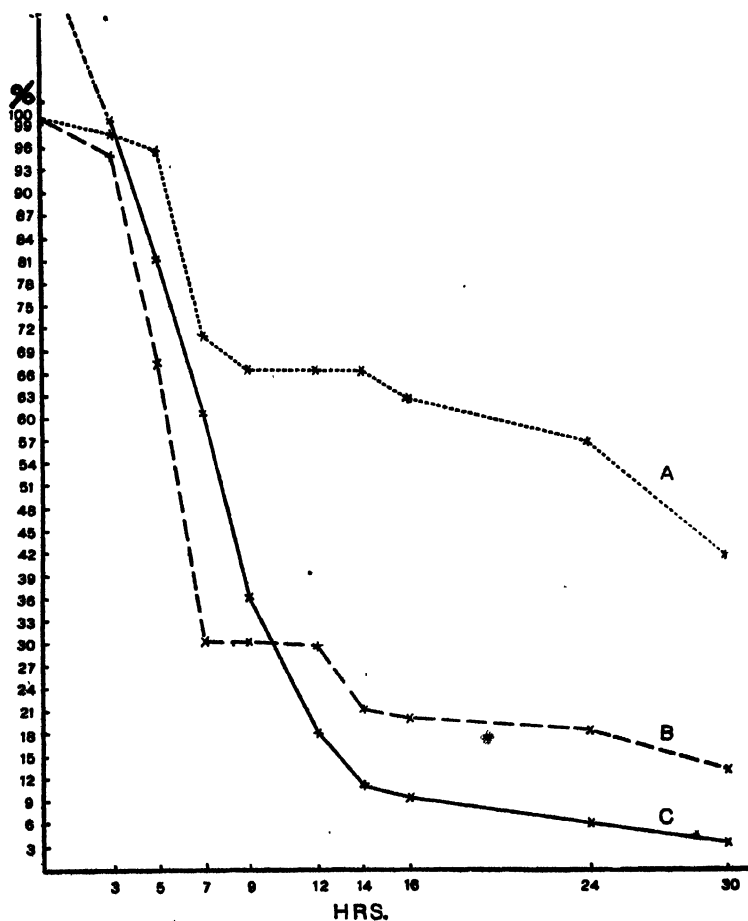


Fig. 8 Curves plotted from percentages in table 7, Experiment VIII, showing the effect of mass or volume of fresh yolk eaten, upon the extrusion reaction. Curves A, B and C are plotted from Sets A, B and C, respectively. The points on the ordinates represent the percentage of total number of grains fed, which still remained in the vacuoles at the time indicated by the same points on the abscissa.

ent, towards the end of the experiment, in the individuals of Sets B and C, was caused by rapid and complete digestion, then the effect of mass on the rate of digestion for fresh yolk would have to be relatively vastly more pronounced with fresh yolk grains than is true for vitellin; and this is not the case.

The first parts of the curves (fig. 8) bring out the points clearly, for here the error, that would be caused by the loss of grains due to complete digestion and not to extrusion, is practically avoided, since the rapid fall in the number of grains takes place during the first part of the experiment, before the digestive process could have been effective in causing total disappearance of the yolk grains; especially is this true for Set C, Curve C. On the other hand, Curve A which we should expect to be markedly affected by digestion, does in fact show only a slight fall, compared to that of Curves B and C. It will be noted that the amounts of fall of the curves show the same *sequence* as the number of grains fed.

The results therefore actually demonstrate beyond question, that the *quantity* of fresh yolk eaten is a determining factor in the process of extrusion. The greater the mass or volume the more effective is the stimulus from the contents in the vacuole. *A quantitative or intensity factor as well as a qualitative factor therefore enters and determines whether or not extrusion of fresh yolk shall take place.*

Now from the results of Experiment VIII we have as yet no evidence showing in what way the mass or volume affects the process; i.e., whether it is an intensity effect due to chemical or mechanical stimulus or both.

Another important fact in connection with the extrusion reaction is that the stimulus from the contents of the vacuole is most effective in bringing about extrusion during a rather limited period (4 to 6 hours with fresh yolk grains) after the formation of the vacuole. This will be seen from the sudden drop in the curves in figure 8, with subsequent tendency to retention of the remains of the yolk. There is a process of functional adjustment (loss of irritability?) with the continued action of the stimulus from the contents of the vacuole. Roughly it may be ex-

pressed by saying that a reaction by digestion is substituted for one by extrusion. These facts appear in a clearer way when the course of extrusion is studied in single individuals.

A further analysis of these responses must be kept for a future time, since the data from other experiments in which were used other substances are not sufficiently complete.

SUMMARY

1. A quantitative method (p. 3) was worked out, by means of which it was possible to study the processes in the food vacuole of *Bursaria*.

2. Yolk and vitellin may be drawn upon as food for the energy requirements and growth of *Bursaria*.

3. The liquid of the newly formed food vacuole is partly made up of the external medium, and partly of an acid secreted by the base of the buccal pouch. After a few minutes this liquid is resorbed and the vacuole membrane becomes applied to the yolk grain. The vacuole contents remain acid in reaction throughout the process of digestion of vitellin and yolk grains.

4. Sooner or later after the initial resorption of liquid about the grain, digestion begins. Digestion may or may not result in the second appearance of liquid in the vacuole, according to the principle that whenever the rate of solution—this perhaps in part depending upon the concentration of the cleavage agent—is greater than the rate of resorption, then the liquid products of digestion accumulate more or less about the grain, while if the rate of solution of the grain is slower than the rate of resorption, then the products of digestion are removed as fast as they are formed. Equilibrium between these processes in the vacuole may be established during digestion of vitellin with much, little, or no liquid present in the vacuole.

5. The average time for complete digestion of vitellin in *Bursaria* was found to be directly proportional to the square root of the quantity of vitellin eaten, i.e., the relation expressed by Arrhenius' formula $t = k\sqrt{M}$ was found to hold to within the limits of experimental error.

6. Congo red adsorbed by vitellin grains and fed to *Bursaria* interferes with or prevents digestion of the parts of the vitellin grain to which the dye has been adsorbed and causes an early extrusion.

7. Olein is digested and resorbed by *Bursaria* while paraffin oil is not affected. Lipoids and fats play an important rôle in promoting growth in *Bursaria*. No evidence was obtained for the formation of stainable lipoid from pure vitellin.

Starch or amyllum grains are not digested.

8. The time of extrusion is determined by the quality (chemical) and the quantity or intensity (chemical, physical or both) of the stimulus from within the vacuole by the substance eaten.

9. The maximum tendency to respond by extrusion to the stimulus from the vacuole contents, exists within a limited time (4 to 6 hours with fresh yolk) after feeding.

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PLATE 1

EXPLANATION OF FIGURES

Typical stages in the process of digestion and resorption in a food vacuole of *Bursaria* containing a single grain of fresh yolk or vitellin.

A Degree of acidity attained by the vacuole as shown by litmus at time of separation from the end of the gullet. The pink edges indicate that acid has been secreted from wall of gullet; figures B and C, further stages in same process.

A to E, inclusive, show course of resorption of liquid enclosed during formation of vacuole.

G to L, inclusive, typical stages of digestion and resorption of fresh yolk (protein and lipoid).

G and H' to L', inclusive, typical stages in digestion and resorption of vitellin-liquid present in the vacuole.

G and H'' to L'', the same as H' to L' except that products of digestion are resorbed as soon as they are formed. Change indicated by figures under x lasted on the average 38 seconds; change shown by figures under y about $4\frac{1}{2}$ to 6 minutes.

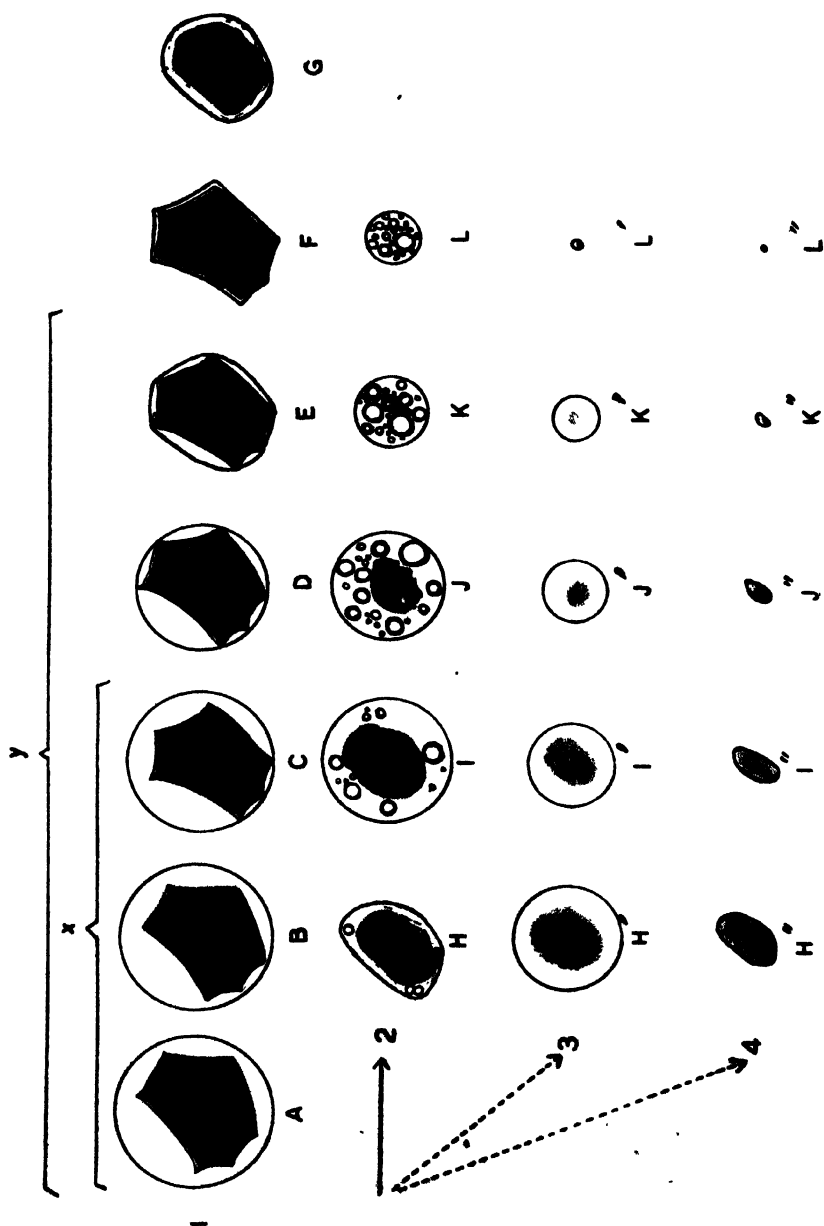
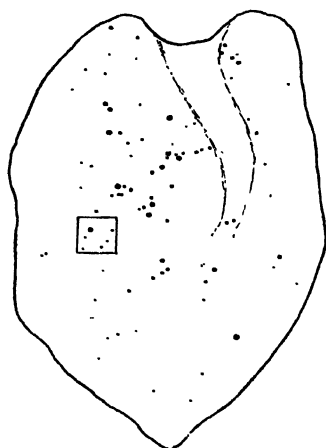


PLATE 2

EXPLANATION OF FIGURES

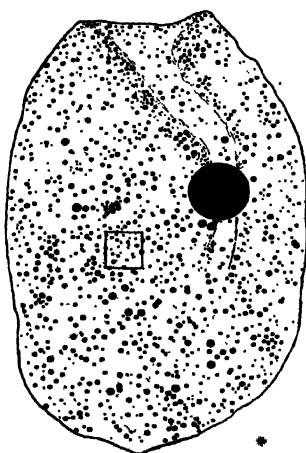
A and B Low power, camera lucida drawings of two sister cells stained to show fat 42 hours after separation. Cell A was not fed; cell B was fed olive oil 18 hours after separation from sister cell. The large drop of oil in *B* represents that remaining in the vacuole. *a* and *b*, high power, camera lucida drawings in one focal plane of area indicated by squares in *A* and *B*, respectively.



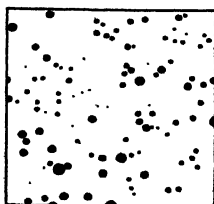
A



a



B



b

CHROMOSOME STUDIES IN THE DIPTERA

I. A PRELIMINARY SURVEY OF FIVE DIFFERENT TYPES OF CHROMOSOME GROUPS IN THE GENUS *DROSOPHILA*

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DIAGRAM AND TWENTY-SIX FIGURES (PLATE)

To the student of chromosomes and chromosome behavior a cytological study of the Diptera presents many features of interest. Some of these have been described or mentioned by Miss N. M. Stevens in connection with her work on the sex chromosomes,¹ but aside from this I know of no serious attempts at such a study. When compared with the detailed and critical works on the chromosomes of the Hemiptera, Orthoptera, and Coleoptera, this lack of knowledge of the Diptera is surprising, and can be explained only by the fact that the latter are in many ways unsatisfactory objects for cytological study. The difficulties in such a study are admittedly numerous, but notwithstanding, much can be accomplished, and the interest of the questions involved seems to me to more than justify the extra effort expended in their investigation.

For this reason a series of such studies has been undertaken, the extent of which will depend upon the time and facilities available. Some are under way at present and others will be taken up subsequently. The results I hope to present in a series of papers, to which the present is introductory.

¹ So far as known to the writer, the papers of Dr. Stevens are the only ones dealing with the chromosomes of the Diptera. They are four in number: The chromosomes of *Drosophila ampelophila*. 1907. Proc. VII Internat. Zoöl. Cong. A study of the germ cells of certain Diptera, with reference to the heterochromosomes and the phenomena of synapsis. 1908. Jour. Exp. Zoöl., vol. 5, pp. 359-374. The chromosomes in the germ cells of *Culex*. 1910. Jour. Exp. Zoöl., vol. 8, pp. 207-217. Further studies on the heterochromosomes of the mosquitoes. 1911. Biol. Bull., vol. 20, p. 109.

The object of this preliminary paper is to examine briefly the chromosomes of several species belonging to the genus *Drosophila*. This is a widely distributed genus and contains a large number of species, of which the most common is the ordinary 'pomace fly' or 'banana fly,' *Drosophila ampelophila*, already well known in genetic circles from the experimental work of Morgan and his students. Aside from *D. ampelophila*, about a dozen other species have been reared in the laboratory and studied cytologically. In the task of obtaining, breeding and identifying the flies I have been greatly assisted by Mr. A. H. Sturtevant, who has shown an active interest in the progress of the work from the beginning, and has kindly furnished cultures of several species which could not otherwise have been obtained. Some of the species studied have apparently never been described by taxonomists, and therefore, cannot be referred to by name in this paper. Consequently they are designated Species A, B, C, etc.

Among the twelve different species examined five distinct types of chromosome groups have been found,² each of which is characteristic of one or more species. These types, when compared with one another, chromosome by chromosome, show a relationship, which, if the hypothesis outlined below be true, may indicate an evolution of chromosomes in the genus. At all events, there is here presented a series of related chromosome groups more complete than has been found in any other genus of animals or plants known to me.

Owing to peculiarities of the male *Drosophila*, it has been necessary to take the data for this study principally from female specimens. The males, unlike those of most insects, are much less satisfactory for chromosome studies than the females, and therefore have been reserved for separate treatment. The male chromosome groups of two or three species are included, as mentioned in the text, but otherwise only the diploid female groups are considered. The latter are sufficient for present purposes, since only the normal somatic divisions are involved, and in these

² The chromosomes of one species, *D. ampelophila*, have been previously described by Dr. Stevens, ('07, '08).

it may be assumed that both sexes agree, save as regards the two sex-chromosomes (heterochromosomes).

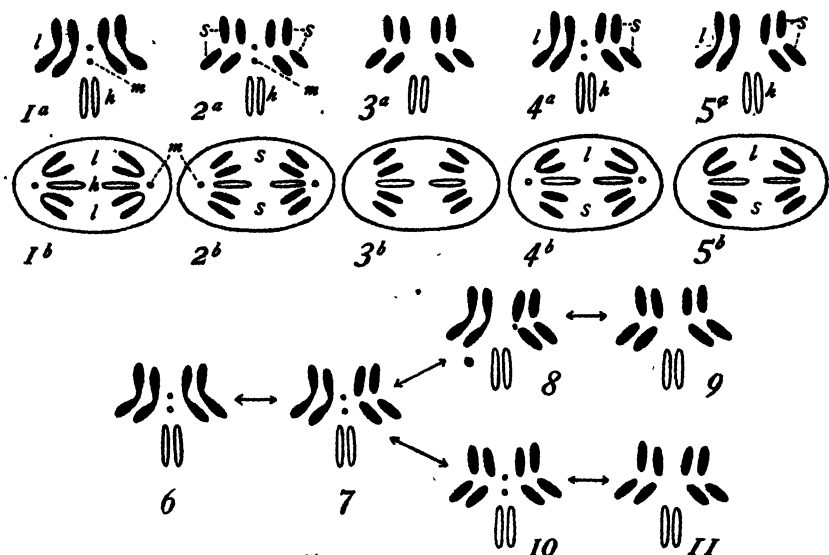
No attempt is made in this paper to elaborate the data. That can be done only after a more extensive study, and one involving more species. Likewise the figures have been limited to a number sufficient merely to illustrate the essential facts.

TYPE I

Chromosome groups found in Drosophila amoena, D. ampelophila, D. quinaria, and two species as yet unnamed

Figures 1 to 5 show metaphase plates of chromosome groups designated as Type I. Figure 1 is a diploid group of *D. amoena* female. Figures 2 and 3 are corresponding groups from males of the same species, introduced here for reference. In figures 4 and 5 similar groups are shown for *D. ampelophila* females. Figure 1 is from a section and indicates the normal relations of the chromosomes, except that one member of the small pair is hidden. The other four are from aceto-carminic smear preparations, and consequently show the chromosomes somewhat out of place or disarranged. Yet the essential similarity of all five figures is quite evident, and in each one the same constellation of chromosomes is readily seen. It is this constellation that is termed Type I.

• If now, we examine any one of the figures, for instance figure 5, we immediately distinguish three different sorts of chromosomes on the basis of differences in size and form. The first sort is very small and spherical (fig. 5, *m*), the second elongate, rod-like or curved (fig. 5, *h*), and the third very long, more or less V-shaped and constricted in the middle (fig. 5, *l*). As a rule these chromosomes are definitely associated in pairs, there being in this group one pair of the first sort, one of the second, and two of the third—four pairs in all. The three classes, separated thus according to size and shape, are also distinguished by characteristic differences in behavior, which exhibit themselves in metaphase, anaphase and prophase. Upon this basis they may be named and characterized as follows:



Diagrams 1 to 5 Diagrams 1 a, 2 a, 3 a, 4 a, 5 a represent metaphase plates of Types I, II, III, IV and V respectively. Diagrams 1 b, 2 b, 3 b, 4 b, 5 b represent anaphases of the same. In these anaphases, for the sake of simplicity, only one member of each chromosome pair is figured; *m.*, microchromosomes; *h.*, sex- or heterochromosomes; *l.*, large autosomes; *s.*, small autosomes.

Diagram 6 to 11 The same types of chromosome groups as above, but arranged in a dichotomous series according to their relationship to one another, and their divergence from Type I. 6, Type I; 7, Type IV; 8, Type V; 9 and 11, Type III; 10, Type II. As the diagrams indicate, Type III may be derived from either Type V or Type II.

1. The *m* or *microchromosomes*.³ One pair; small, spherical; usually occupying the center of the plate in metaphase; going to the poles as short, straight rods in anaphase (*m*, diagrams 1 a and 1 b).

2. The *sex-chromosomes* or *heterochromosomes*. One pair; unequal in the male,⁴ equal in the female; elongate, rod-like,

³ A name first applied by Wilson to a similar small pair seen in many coreid Hemiptera; (E. B. Wilson, *Studies on chromosomes*. II. 1905. *Jour. Exp. Zool.* vol. 2, pp. 508-545). Whether the pair in *Drosophila* is strictly comparable with these in the Hemiptera is not certain, and I have used the term here merely for convenience. It remains to be seen how the pair behaves in the maturation stages.

⁴ This inequality in the male was first pointed out by Miss Stevens ('08) in the case of *D. ampelophila*. In so far as the diploid chromosome groups of this species

usually lying radially in metaphase, i.e., one end pointing toward the center, the other toward the margin of the plate (diagram 1 a, *h*); attached to the spindle fiber at *one end*, and going to the poles as elongate straight rods, (*h*, diagram 1 b, and fig. 6); contracting precociously in prophase.

3. The *large autosomes*. Two pairs (in Type I); very long, more or less dumb-bell-shaped, with a definite constriction in the middle (*l*, diagram, 1 a and fig. 5); usually V- or U-shaped in metaphase, the apex of the *V* coinciding with the constriction and pointing toward the center of the plate, thus bringing the arms of the *V* into the position of radii pointing toward the margin; attached to the spindle fiber, not at one end, but at the *median, constricted part*, and going to the poles in V-shape (*l*, diagram 1 b and figs. 6 and 7).

The essential features in the behavior of these different sorts of chromosomes are indicated in figures 1 to 7 and diagrams 1 a and 1 b. Figure 7 shows an anaphase in polar view illustrating the difference between the V-shaped, large autosomes and the straight sex-chromosomes. Only one pole is figured, the other being almost identical with it. In this figure the *m*-chromosomes are not evident. Figure 6 shows the same features in side view. It is taken from the second maturation division of the male, involving only the haploid number of chromosomes (4), and although one pole is somewhat confused the figure shows the decided difference between the two kinds of chromosomes (*l* and *h*).

are concerned, my observations are entirely in accord with hers. But in the maturation divisions, where she describes a distinct *X* element separable from the *Y* of which it was formerly an integral part, I fail to corroborate her statements. So far as my observations go, they indicate an unequal *XY* pair in the male, without any additional piece attached to either. Neither my observations nor those of Miss Stevens are conclusive, however, owing to the difficulty of observing the chromosomes in these stages. The question is important for the bearing it has upon the breeding experiments with this fly, and we are doubly unfortunate in being thus far unable to settle it. By a recent examination of males of another species, having the same type of chromosomes as *ampelophila*, and showing more favorable conditions for their study, I have been led to hope that through this species some light can be thrown on *ampelophila*.

TYPE II

Chromosome groups found in Drosophila repleta, Species A and Species B

The type of chromosome group exhibited by these three species differs very decidedly, at first sight, from that just studied. It may be best understood by examining diagram 2 (a and b) and figures 8 to 13. The figures in this case are all but one (fig. 9) taken from fixed and sectioned material, and therefore show more nearly the proper arrangement of the chromosomes. Examining the individual chromosomes, as was done in Type I, two kinds found in that type are seen to be present here also. These are the small *m*-chromosomes and the sex-chromosomes (in this case the longest pair), as indicated in diagram 2. In addition to these two kinds there remain four pairs of short, rod-like autosomes in place of the two pairs of long, dumb-bell-shaped large autosomes of Type I. These constitute a fourth kind of chromosomes and may be characterized as follows:

4. The *small autosomes*. Short, rod-like autosomes without any median constriction; almost invariably straight in metaphase, and lying in a radial position, i.e., pointing from the center toward the margin of the plate (figs. 8, 10, 12); attached to the spindle fibers at one end, thus going to the poles as straight rods (s, diagram 2 a and 2 b).

The principal interest of Type II as compared with Type I centers around these eight small autosomes, which, it appears, replace the four large autosomes of Type I. This latter fact at once suggests the idea that each of the large autosomes has parted in the middle, at the point of constriction, and has thus given rise to two small ones. In this way the four large dumb-bell-shaped chromosomes could have been readily transformed into the eight small ones. The evidence of size, form, and behavior of the two kinds supports this view in a rather surprising manner. Diagram 2 (a and b) are designed to show this relationship schematically. They are purposely made very schematic, but the essential features are entirely substantiated by actual division figures. The

significant fact is that each of the small autosomes resembles in size, form and behavior one arm, or one end of a large autosome. So that by assuming a partition of the large autosomes at their median, constricted points, and assuming that each of the resulting halves behaved after separation just as it did before, exactly such a condition would be brought about as that found in Type II.

TYPE III

Chromosome groups found in Drosophila funebris

The important features in this type are shown in figures 14 to 17, and diagram 3 a and 3 b. In the main it agrees essentially with Type II except that the *m*-chromosome pair appears to be entirely lacking (compare figs. 8 and 14). Whether this disappearance has been brought about by the degeneration of the pair or by a fusion with some other pair I have been unable to determine. The unusual length of the sex-chromosomes might suggest the latter, but in view of the radical difference in behavior between them and the autosomes, and the fact that another type (V) also lacks the *m*-chromosomes, without a corresponding enlargement of the sex-chromosomes, I am inclined to doubt this explanation.

It is worthy of note that in this type (III) the sex-chromosomes are fully as elongate as the large autosomes of Type I, yet they retain their own characteristic form and behavior, and show no evidence of the V- or dumb-bell-shape, or a median attachment to the spindle fibers. If it were true that all chromosomes are qualitatively alike, and that form and behavior are merely secondary characteristics determined by size and bulk, then we should expect these long sex-chromosomes to assume the characteristics of the large autosomes of Type I, but as a matter of fact they do nothing of the sort. To my mind there is no clearer demonstration than this of a qualitative difference between the individual chromosomes of a group.

TYPES IV AND V

*Chromosome groups found respectively in Species C
and D. tripunctata*

Like Types II and III, these two differ from one another mainly in the presence and absence of the *m*-chromosomes. Otherwise they are essentially alike, and may be considered together. Figures 18 and 19 show early metaphases of Type IV taken from aceto-carmine smear preparations. Figure 20 shows the haploid number of the same, in the second spermatocyte division (equation division), from a section. In figures 21 to 26 are shown the diploid chromosomes of Type V, taken from sections. The first two figures represent metaphases—21 a polar view, and 22 a somewhat diagonal view. They differ in proportions from the figures of Type IV because of the contraction incident to fixing, embedding, etc., but nevertheless show their general similarity to the other type; (compare figs. 18 and 22). Figure 23, a late prophase of Type V, represents an earlier stage, before the autosomes have fully contracted. Figure 24 is an early prophase, included here to show the precocious contraction of the sex-chromosomes, and the very intimate pairing or conjugation of the autosomes. In figures 25 and 26 are shown the two poles of an anaphase, indicating the behavior of the three different kinds of chromosomes at this stage.

These two types (IV and V) are less easily analyzed than the previous ones, but the evidence clearly indicates that they are, in a way, intermediate between certain of the latter. Comparing Type IV with Type I, as indicated in diagrams 4 and 1, it would seem that two of the large autosomes of Type I have undergone partition, giving rise to four small autosomes, while the other large pair has remained intact. By a similar partition of the latter a type like number II would be formed. From Type IV one like Type V could be derived by loss of the *m*-chromosome pair, as assumed in the case of Type III, either by degeneration, or by fusion with another pair. These various steps are indicated schematically in diagrams 6 to 11.

The principal difficulty encountered in the examination of Types IV and V, is that of distinguishing between the sex-chromosomes and the small autosomes. As shown by the figures, the likeness is so great that at certain stages they cannot be distinguished with certainty. This causes some trouble in late prophase and early metaphase, when the autosomes are in the process of contraction, but before and after this the separation may be made readily, so that the difficulty in no way interferes with the identification of the various chromosomes, or with the process of homologizing them with members of other types.

SUMMARY AND CONCLUSIONS

The genus *Drosophila* has numerous species which differ from one another in the number and form of their chromosomes, as well as in external characters. Of the twelve species examined by me five conform to one type in respect to the chromosomes, four conform to another type, and three to three additional types respectively—making five types in all. In each type the chromosome group is made up of certain different kinds of chromosomes, distinguished by their size, form and behavior. By comparing these types with one another an evolutionary series may be arranged. This series can be explained upon the assumption that four of the types arose from the first by a series of steps, involving, on the one hand, the partition of certain long chromosomes into halves, and on the other hand, the disappearance (by degeneration or fusion), in two cases, of a pair of very small *m*-chromosomes. It is not likely that the forms studied represent a consecutive series. In themselves they make a dichotomous series, but in all probability the intermediate steps in this constructed series, if they represent evolutionary stages at all, are merely indications of what has taken place, and do not themselves belong in the positions assigned to them.

Type I is arbitrarily chosen as the stem from which the others are derived because it includes the greatest range of species, and because it is easier to conceive of an evolution by partition, or by loss, of chromosomes than by their fusion or generation. The

direction of evolution, however, is purely a matter of opinion and may quite readily be reversed.

* As far as the characters of the insects themselves are concerned, they throw little light on the question. The various species are, for the most part, quite different from one another, with the exception of two, and these have similar chromosomes (Type I). From the taxonomic standpoint the genus has never received adequate attention from entomologists, and consequently there is little clue as to the relationship of species.

DISCUSSION

A detailed discussion of the various questions suggested by this study would be out of place at this time, but it may be profitable to call attention briefly to some phenomena appearing in these flies, which do not present themselves so conspicuously in other animals.

Individuality of the chromosomes

In practically all the species used in this study the metaphase plates stand out as clearly as diagrams, and in most cases there cannot be the slightest doubt as to the identity of any particular kind of chromosomes. Especially is this true of such types as II and III, in which most of the chromosomes are short. And even in the other types where more long chromosomes are present there is no difficulty in identifying the different sorts except at certain stages. Consequently, after studying scores of preparations from larvae, pupae and adults, and finding that in each of these the same particular kinds of chromosomes appear over and over again with the greatest regularity, both in the somatic and germinal tissues; and then after noting that each of these kinds has its own particular method of behavior in each cell division, I can find little reason to doubt that every chromosome has a specific character of its own, and that its individuality persists from one generation to another. Upon no other assumption can I explain the exact repetition of identical chromosome groups in certain species, and the appearance in other species of groups so related to one

another and to the first that the individual chromosomes can be homologized throughout the entire series, as they can be in the *Drosophilas*.

Pairing and conjugation of chromosomes

The *Drosophilas* offer some of the most striking evidences of the actual pairing of chromosomes in the diploid groups, thus far observed. The phenomenon is apparently characteristic of all Diptera,⁵ but is nowhere so striking as in this genus.

The figures speak for themselves in this regard, and need little emphasis. The sex-chromosomes alone show any appreciable number of exceptions to such a paired association. A number of cases have been found in which these were not paired, and in general the association between them is less intimate than that between members of autosome pairs. Whether this is correlated with the precocious contraction of the sex-chromosomes, or is due to an inherent difference between them and the autosomes, cannot be determined. In any event, the difference is slight, for normally they are paired like the autosomes.

In such cases as these, where two or three pairs of chromosomes can be individually identified, there can be no question that *each pair is composed of one maternal and one paternal component*, since any germ cell of either sex contains only one member of each of these kinds. And when it is evident that three out of five pairs are so made up (e.g., Type IV), a very strong presumption is created that the other two pairs are similarly constituted.

But the most remarkable feature of the whole study is the discovery that the chromosomes not only exhibit a close association in pairs at nearly all times, but that before every cell division the members of each pair become so intimately united that they may be said actually to conjugate. Each pair, with the possible exception of the sex-chromosomes, goes through what amounts to a *synapsis* in every cell generation, so that in many cases the

⁵ Miss Stevens ('08, '11) records it in nine species of Muscidae, and four species of mosquitoes. I have verified it in five of these species of Muscidae, and extended it to eight others, in addition to those of *Drosophila*.

figures closely resemble those of haploid groups. Apparently this takes place especially in early prophase (fig. 24), but a second conjugation may occur during metaphase, just a short time before division (figs. 9, 15, 16). Whether in the first case conjugation takes place in anaphase and continues into the succeeding prophase has not been ascertained. This interesting question is being given special study at the present time, with a view to determining the exact nature and extent of the conjugation. In the second, or metaphase conjugation, at least, it is worthy of note that the union is unquestionably a side-by-side, or *parasynaptic* one.

It need hardly be urged, in the face of such evidence as this, that an actual pairing of chromosomes may take place in the diploid nuclei outside of the sphere of reduction. True, there is little evidence, in most organisms, of such a side-by-side association of chromosomes to form pairs. But the fact that it occurs so strikingly in some forms adds great weight to the conclusion (based on size relations and other evidence), that such pairs are present in all, even when the members are not obviously associated. If anyone still doubts the reality of pairing of homologous chromosomes, I should recommend to him a study of the Diptera.

• In conclusion, I wish to express my appreciation of the kindly assistance and advice of Professors E. B. Wilson and T. H. Morgan of this laboratory. And I wish to thank Dr. C. B. Davenport for the many courtesies extended to me during my stay at Cold Spring Harbor.

Addendum. Since this paper went to the publisher, further study of *D. funebris* (Type III) has revealed the presence of a minute pair of *m*-chromosomes in at least some specimens of this species. Apparently either the size of the *m*-chromosomes is variable in *D. funebris*, or else these chromosomes are absent in some specimens and present in others.

PLATE

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PLATE I

EXPLANATION OF FIGURES

All figures, with the exception of figure 9, were drawn with the aid of a camera lucida, using Zeiss 1.5 mm. apochromatic objective, and compensating ocular 12, with tube length of 160 mm. The drawings are reproduced natural size. Figure 9 was drawn freehand, but to about the same scale as the others. For the preparation of figures 6, 9, 17, 23 and 24 I am greatly indebted to Miss Mabel L. Hedge. Figures designated as 'oogonial' may be, in some cases, from ovarian follicle cells; the two cannot always be distinguished.

1-7 Chromosome groups of Type I.

1 *Drosophila amoena*, oogonial metaphase; from a section (one *m*-chromosome concealed).

2 and 3 *D. amoena*; spermatogonial metaphases; aceto-carmin.

4 and 5 *D. ampelophila*; oogonial metaphases; aceto-carmin.

6 *D. amoena*; second spermatocyte anaphase; side view; showing haploid number of chromosomes; section

7 *D. quinaria*; one pole of anaphase; oogonial division. Polar view showing contrast between large autosomes and sex-chromosomes; *m*-chromosomes not shown; section

8 to 13 Groups of Type II.

8 *D. repleta*; oogonial metaphase; section.

9 *D. repleta*; oogonial metaphase slightly earlier than figure 8, showing conjugation of chromosomes; aceto-carmin.

10 *D. (Species A)*; oogonial metaphase; section.

11 *D. (Species B)*; early metaphase or late prophase; section.

12 and 13 Same; later stage; sections.

14 to 17 Type III.

14 and 15 *D. funebris*; metaphase from ovarian cells; sections.

16 *D. funebris*; earlier than last, showing conjugation of autosomes; section.

17 *D. funebris*; anaphase, polar view showing both poles; All chromosomes rod-like; the autosomes completely divided and daughter halves separated; the sex-chromosomes just pulling apart, but still attached at one end; section.

18 to 20 Type IV.

18 and 19 *D. (Species C)*; oogonial metaphase; aceto-carmin.

20 Same; second spermatocyte metaphase; chromosomes partly divided; section.

21 to 26 Type V.

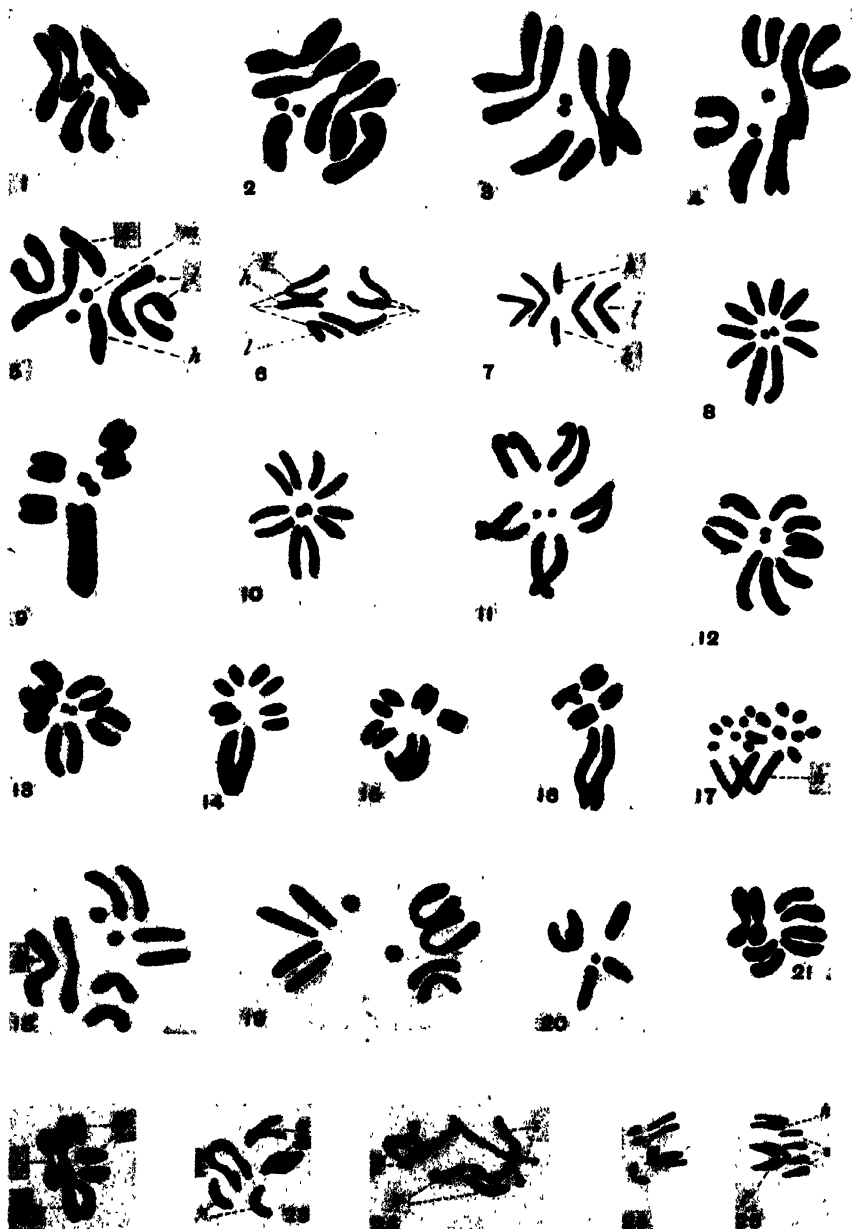
21 *D. tripunctata*; polar view of metaphase from ovarian cell; section.

22 *D. tripunctata*; diagonal view of metaphase; slightly later stage; section.

23 *D. tripunctata*; late prophase from ovarian cell; section; *h*, sex-chromosomes.

24 *D. tripunctata*; early prophase from ovarian cell; autosomes conjugated in pairs; section.

25 and 26 *D. tripunctata*; two poles of anaphase from ovarian cell. Show V-shaped large autosomes (*l*), rod-like small autosomes (*s*), and rod-like sex-chromosomes (*h*), section.



STUDIES ON THE DYNAMICS OF MORPHOGENESIS AND INHERITANCE IN EXPERIMENTAL REPRODUCTION

VIII. DYNAMIC FACTORS IN HEAD-DETERMINATION IN PLANARIA

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TWO FIGURES

In earlier papers (Child '11 b, '11 c, '11 e) attention has been called to the fact that the frequency of head-formation in isolated pieces of *Planaria dorotocephala* varies with size of piece, region of body and various external conditions which can be controlled experimentally. This difference in capacity, together with the possibility of comparing rates of metabolism in different pieces by the susceptibility method (Child '13 a), affords a means of determining why some pieces produce a head and others do not and since the formation of the head is the first step in the development of the new individual we have an answer to the question why some pieces give rise to new wholes and others do not.

I. THE TIME OF HEAD-DETERMINATION

Since it can be determined experimentally within wide limits in various ways whether a piece of *Planaria dorotocephala* shall give rise to a head or not (Child '11b), it is evident that the fate of the piece as regards head formation is not fixedly and finally determined at the time of isolation of the piece from the parent body. The first step in the analysis of the factors concerned in head determination is to find when the determination of the head occurs. This can be readily and very simply done by the following method.

Short pieces from the middle region of the body, i.e., the posterior region of the first zooid of *Planaria dorotocephala*, such for example, as *ab*, *bc* and *cd* in figure 1, give rise in 98 to 100 per cent of the cases, under ordinary conditions, to headless forms (Child

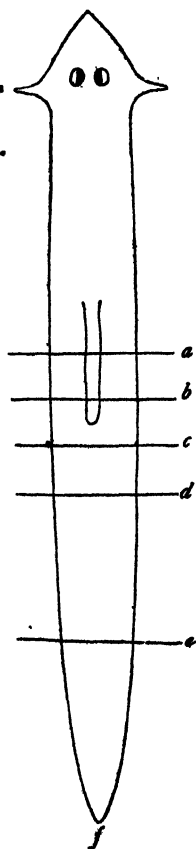


Figure 1

'11 b, '11 c), i.e., no outgrowth of new tissue occurs at the anterior and beyond the healing of the wound.

On the other hand, long pieces with anterior ends at the same levels as those of the short pieces (pieces like *ae*, *af*, *ce*, *cf*) produce under the same external conditions 98 to 100 per cent of animals with fully developed normal heads.

It is of course evident that in these cases the length of the piece is in some way a factor in head-formation, but the question how it affects head-formation will be answered later. At present we are concerned merely with the fact that the cells at the levels, *a*, *b*, etc., when at the anterior ends of short pieces, do not give rise to heads, but when at the anterior ends of long pieces do give rise to heads. In order to find approximately when head-formation is determined, it is only necessary to prepare a large series of the long pieces, such as *af* or *cf* in figure 1, and at different intervals after section to remove short pieces such as *ab* or *cd* from the anterior ends of a certain number. We know that such short pieces when completely isolated at once do not develop heads. If heads appear at their anterior ends after they have remained a certain length of time as the anterior regions of long pieces and have then been isolated, it is evident that the formation of heads must have been determined while they were still a part of the long piece. The records of experiments will show the character of results obtained.

. *Series 458.* From well fed worms 18 mm. long, about 150 pieces, including the region *cf* in figure 1, were prepared and from these the following five lots were taken.

Lot I. From the anterior ends of twenty-five of the long pieces, short pieces like *cd* were removed 15 to 30 minutes after the long pieces were prepared.

Lot II. From another twenty-five of the long pieces, similar short pieces were cut 3 to 4 hours after the long pieces were prepared.

Lot III. Twenty-five similar short pieces 7 to 8 hours after first operation.

Lot IV. Twenty-five short pieces 18 hours after first operation.

Lot V. Twenty-five short pieces 24 hours after first operation.

These five lots of short pieces were allowed to undergo reconstitution at a temperature of 20 to 22°C. and when this was complete the character of each piece was recorded; the results appear in table 1.

The five types distinguished have been described in earlier papers (Child '11 b, '11 c). The normal form possesses a head

with two eyes and two lateral cephalic lobes, as in figure 1. In the teratophthalmic form the eyes are either partially or wholly united or unequal in size or abnormally placed. The teratomorphic form has a single eye in the median line and the cephalic lobes appear on the front of the head and may be partially or completely united in the median line. In the anophthalmic form an anterior outgrowth is present, containing a small abnormal ganglionic mass, but eyes do not appear. And finally, the headless form shows no anterior outgrowth except closure of the wound.

The increase in head-frequency in the short pieces is evident from table 1. In Lot I, 80 per cent remain headless and only 8 per cent give rise to heads with eyes of any kind and none are

TABLE 1

LOTS	TIME BET. 1ST AND 2D OPERA- TION IN HRS. AND MIN.	NORMAL	TERATOPH- THALMIC	TERATO- MORPHIC	ANOPH- THALMIC	HEADLESS	DEAD
I	0.15-0.30		4	4	12	80	
II	3.00-4.00	4	32	4	24	28	8
III	7.00-8.00	28	60			12	
IV	18.00	76	16			4	4
V	24.00	80	8				12

normal. But Lot II shows that after 3 to 4 hours the anterior ends of the long pieces have been determined as heads to such an extent that their isolation as parts of short pieces prevents head-formation completely in only 28 per cent, while 24 per cent are anophthalmic and the rest form heads with eyes, 4 per cent normal, 32 per cent teratophthalmic and 4 per cent teratomorphic. In Lot III, where the short pieces were cut off after 7 to 8 hours as parts of the long pieces, only 12 per cent remain headless and 28 per cent are normal and 60 per cent teratophthalmic. In Lot IV, after eighteen hours, 76 per cent are normal and 16 teratophthalmic, and in Lot V, after twenty-four hours, 80 per cent are normal. After 18 hours the head is so fixedly determined in practically all cases that isolation of the head-forming region as part of a short piece cannot prevent head-formation. The approach in

the character of the head to the normal, as well as the increase in head-frequency, is also evident. In Lot II only 4 per cent of normal heads appears, but in Lot III 28 per cent are normal, in Lot IV, 76 per cent and in Lot V, 80 per cent. This series shows that head determination begins almost at once after section and that in most pieces, the head is fixedly determined within seven to eight hours after section (Lot III, 88 per cent form heads).

The following series gives in general the same results but brings out some points more clearly than the preceding.

TABLE 2

LOTS	TIME BET. 1ST AND 2D OPERA- TION IN HRS. AND MIN.	NORMAL	TERATOPH- THALMIC	TERATO- MORPHIC	ANOPE- THALMIC	HEADLESS	DEAD
Controls		72	28				
I	0					96	4
II	2.30-3.00	4	16		12	68	
III	5.30-6.00	4	28	8	24	32	4
IV	12.00	36	60			4	

Series 465. From well-fed worms 15 to 16 mm. in length, about 150 long pieces like *ae* (fig. 1) were cut.

Lot I. Twenty-five short pieces (*ab*, fig. 1) cut immediately after first operation.

Lot II. Twenty-five short pieces cut 2½ to 3 hours after first operation.

Lot III. Twenty-five short pieces cut 5½ to 6 hours after first operation.

Lot IV. Twenty-five short pieces cut 12 hours after first operation.

All lots kept at 20 to 22°C. The results appear in table 2, in percentages.

The controls, i.e., twenty-five of the long pieces, all form heads, 72 per cent normal and 28 per cent teratophthalmic. On the other hand, Lot I, consisting of twenty-five of the short pieces removed at once from the anterior end of the long piece, develops no heads at all, 96 per cent being headless and 4 per cent dead.

The contrast in head-frequency between the long and short pieces is strikingly shown in these two lots. It must be remembered that in the two cases the level of the body concerned in head-formation is the same, or as nearly as possible the same, yet in the long pieces 100 per cent of heads are formed and in the short pieces none. It may be added further that in pieces of intermediate length all possible intermediate percentages of head-frequency are found.

Comparison of Lots I to IV of the short pieces shows essentially the same result as table 1 above. In Lot I all remain headless, in Lot II, 68 per cent, in Lot III, 32 per cent and in Lot IV, 4 per cent. The table also shows that not only does the head-frequency increase but that a marked approach to the normal in the character of the head occurs, as the length of time during which the short piece remains as an anterior portion of the long piece increases. In Lot II, 20 per cent of the pieces form heads with eyes, in Lot III, 40 per cent, and in Lot IV, 96 per cent.

The only possible conclusion from these two series is that the factors which determine whether an isolated piece shall give rise to a head or not, begin to act almost immediately after the operation; that within 3 hours after section, the determination between 'head' and 'headless' has already occurred in about 50 per cent of a lot of pieces under the usual conditions and that this determination has occurred in practically 100 per cent within twelve hours after section.

It also appears from the two tables that the determination of the character of the head formed occurs somewhat later than the determination whether a head shall be formed or not. At 3 hours and even at 6 hours, anophthalmic as well as normal and teratophthalmic forms appear in considerable percentages (table 2) and even at 12 hours the percentages of normal heads is only half that in the controls. At 18 hours, however, most heads are determined as normal (Lot IV, table 1).

These and other similar series in which worms of approximately the same size and physiological condition were used and in which external conditions are as nearly as possible uniform, all give similar results. Under these conditions, head-formation

is fixedly determined in most pieces within 8 hours after section and in practically all, within 12 hours after section. The determination of the character of the head occurs later. In this way it is possible to discover approximately the time of determination, not only of the head as a whole, but of the cephalic lobes, eyes and preocular region and of the various types of head. For comparable results animals of similar size and condition and similar external conditions are necessary, because the time of head-determination varies both with internal and external factors and in fact can be altered experimentally.

The times obtained in this way, however, represent the times when the structures concerned have become so fixedly determined that they cannot be altered even by extreme changes in conditions. There is every reason to believe that in a given piece it is determined whether a head shall form or not some time before that determination becomes so firmly fixed as to be unchangeable by altered conditions. Tables 1 and 2 show that in a considerable percentage of pieces, head-determination has become fixed within 3 hours after section. In short, there is no question that under the usual conditions head-determination in pieces must occur, or at least begin, almost immediately after section and the conditions existing in the piece during the first two or three hours after section must constitute the most important factors in the process of head-determination.

II. HEAD-FREQUENCY AND DEGREE OF STIMULATION IN PIECES

In the first paper of this series (Child '11 c) it was shown that the frequency of head-formation in pieces under the usual conditions of temperature, etc., decreases with decrease in length and also with increasingly posterior level of the piece within the limits of a single zoöid (Child '11 e). In other words, the shorter or the more posterior a piece is, the less likely it is to give rise to a head. In the preceding paper (Child '14 b) it was found that the amount of temporary increase in the rate of metabolism, i.e., of stimulation of pieces resulting from section, increases with decreasing length and increasingly posterior level of the piece within the limits of a single zoöid.

Bringing these two groups of facts together, we see that *frequency of head-formation decreases as stimulation following section increases*. This relation seems at first glance somewhat paradoxical, for it means essentially that the lower the rate of metabolism in a piece in general, the more likely it is to give rise to a head, and vice versa, but in this and following papers evidence will be presented to show that this relation holds within certain limits.

It was demonstrated in the preceding section that head-determination occurs within a few hours after section, at the latest, in other words, during the period of stimulation following section, which lasts for several hours and is followed by a gradual fall in rate of metabolism. We are then forced to the conclusion that a relatively high rate of metabolism in the piece as a whole, acts in some way as a factor inhibiting head-formation. This conclusion appears somewhat revolutionary, for it is generally believed that the development of a new head on a headless piece is a process of replacement of a missing part and that it is determined and controlled by other parts of the piece. But if the facts cited above are correct, the more vigorous the piece and the more capable it is of determining and controlling processes in other parts during the period when head-determination occurs, the less frequently does a head arise from it. This can mean only that the maintenance of the piece and the process of head-formation are in some way opposed or antagonistic to each other, and that the new head is not determined by the piece, but rather in spite of it. This point of view has already been briefly stated, together with some of the evidence on which it is based (Child '13 b), but further consideration is necessary to make clear its important features and its significance for our conception of the individual and its development.

III. THE PROCESS OF HEAD-DETERMINATION

The head arises from cells adjoining the anterior cut surface of the piece, which are so greatly affected by the altered correlative conditions and the presence of the wound that they lose their differentiation more or less completely, begin to divide rapidly and produce the outgrowth of new tissue. From this

mass of new embryonic tissue the new head arises. This head then develops from the earliest stages of morphogenesis, while in other regions of the piece, except at the posterior end, the original structure undergoes more or less alteration but does not completely disappear in a regression to embryonic cells. In figure 2 the region of head-formation is indicated by the shaded region at x , the region of tail-formation by z , and those regions of the piece which retain the chief features of their structure, by y .

We have already seen that the higher the rate of metabolism in y following section, the less likely is a head to arise from x , and vice versa, and it has been pointed out that this inverse re-



Figure 2

lation between head-frequency and rate of metabolism in other regions of the piece, can mean only that the new head develops, not in correlation with and under the control of the piece as a whole, but, so to speak, in spite of it.

In short, the facts already cited, and a large body of evidence still to be presented, point directly to the conclusion that head-formation in a headless piece is not the restitution of a missing part but the first step in the development of a new individual. If this is the case, then head-formation in a piece is essentially the same process as in embryonic development. There the head, or more specifically the cephalic nervous system, is the first region or organ of the individual to become morphologically visible. So far as we can determine, these early stages of head-development are not dependent upon conditions in other regions of the egg or embryo, but the head-region takes the lead in development.

In an earlier paper (Child '12) the writer has shown that in *Planaria* reconstitutive development may be inhibited in various degrees by low concentrations of narcotics and that the formation of a head may still occur when all development of other parts is completely inhibited. Moreover, it is a familiar fact that very short pieces of *Planaria* may give rise to a single head or to biaxial heads without other parts, and the same is true for *Tubularia* and various other forms. These various facts demonstrate that head-formation in *Planaria* is possible without any correlative influence of other parts, i.e., it is a process of 'self-differentiation' (Child '13 b, pp. 614-617). On the other hand, there is no evidence favoring the opposite conclusion. Even as regards pieces undergoing reconstitution, there are no facts indicating that head-formation is determined by other regions of the piece. We have simply been accustomed to consider that the piece replaces lost parts and so gives rise again to a new whole. But it has often been pointed out that in such forms as *Planaria*, the piece does not replace exactly the parts lost, but a new whole arises by the formation of a head at one end, a posterior end at the other and the reorganization of remaining portions. What actually occurs in these cases is that a new head region begins to develop as the first step in a new individuation and this new head region dominates other parts and determines a reorganization of the old tissues of the piece. Head-formation is not determined by other parts, but it—or more specifically the formation of the cephalic nervous system—represents the fundamental morphogenetic reaction of the specific cellular material.

The existence in *Planaria* of the inverse relation between head-frequency and rate of metabolism in the piece as a whole, constitutes further important evidence in support of this view and is readily understood from this standpoint. It can mean only that the cells of the region x (fig. 2) give rise to a head except in case the rate of metabolism in the region y is sufficiently high to retard or inhibit this process of "self-differentiation."

The rate of metabolism in the cells at x , which we may call 'rate x ,' is probably determined largely by local conditions connected with the altered correlative conditions and the presence

of the wound. Under given external conditions, it probably does not differ very greatly in pieces of different length and from different regions. The rate in the region y (rate y), on the other hand, varies, as we have seen, with size and level of body. When rate y is sufficiently high in relation to rate x , the cells at x are prevented from beginning independent development and producing a new head. On the other hand, the lower the rate of y in relation to the rate of x , the more independent are the cells at x and the more likely to produce a head.

The fact that head-determination in pieces occurs almost immediately after section, shows that the critical period is at the very beginning of the division and growth in the region x . Evidently, the determination whether a head shall arise or not is essentially simply a question whether the region x shall develop with at least a certain degree of independence of the region y , in which case it produces a head, or whether its development shall be inhibited by y .

If this conception of the process of head-determination is correct, then we arrive at a very simple expression for the head-frequency in pieces of different size and from different regions, viz., head-frequency = $\frac{\text{rate } x}{\text{rate } y}$

We do not know positively whether rate y must actually be higher than rate x in order to inhibit head-formation, but there is no reason to believe that such a difference is necessary. It has been pointed out in earlier papers (Child '11 d, '12, '13 c) that in the intact animal the rate of metabolism decreases from the head region posteriorly. If this is the case and if the effects of section and the local effect of the wound could be eliminated, rate x in a piece should always be higher than rate y . But the region y is a system of correlated parts with conducting paths and metabolic mechanisms fully developed and capable of a relatively high degree of stimulation. The region x , on the other hand, during the first few hours after section (i.e., the period when its fate is determined) is merely a group of cells without definite mechanism of correlation corresponding to a head. It is possible that the fully developed region y may overbalance and inhibit

the few cells at x , even though rate y per unit of weight or volume is not actually higher than rate x . The development of conducting paths, and in general the differentiation of y , must render it more capable of producing correlative effects upon other parts than are the few cells which are undergoing dedifferentiation at x . Probably the rate of metabolism, cell for cell, is higher in x than in y or becomes so very soon after section, for the cells at x are those most affected by section. Susceptibility experiments indicate that the region x usually possesses the highest rate of any part of the piece. But in order to be able to develop in spite of the region y , rate x must in all probability be considerably higher than rate y , and the differences in susceptibility of the regions x and y indicate that this is actually the case. In fact, before the new tissue has developed far enough to permit the distinction between heads and headless forms, we find in general a greater susceptibility in the region x as compared with y , in those pieces which would later show the higher head-frequency.

But whatever particular relation between rate x and rate y which may prove to be necessary for the inhibition of head-formation or the development of a head, the expression head-frequency = $\frac{\text{rate } x}{\text{rate } y}$ still serves to indicate the internal conditions which influence head-formation. And not merely head-frequency in general but the frequency of any of the different types of head, normal, teratophthalmic, teratomorphic, anophthalmic, is determined in the same way. It will be shown that all these different types of head represent simply different degrees of retardation of the process of head-formation, whether by external or internal factors. In very short pieces, where the whole or nearly the whole piece represents region x and region y is absent or very small, head-frequency should become proportional to rate x , and this is actually the case. Any conditions which decrease the rate sufficiently decrease the frequency of head-formation. This point will be considered more fully at another time. If the region x once succeeds in beginning its independent course of development it soon becomes the dominant region of the piece (Child '11 d), develops into a head and determines the establish-

ment of a new axial gradient and so the reorganization of the region y .

As regards the region z of figure 2 from which the posterior end develops, the relations to other parts are not as clear as in the case of x , for the chief visible differences in tail-formation are merely differences in amount of growth. In general, however, it is evident that development of the posterior end is directly proportional to rate y . But the stimulation following section has no relation to tail formation, because it is merely temporary and the tail is a subordinate part, depending for its very existence upon correlations with more anterior regions. It can form at any time, whenever rate y is high enough to determine its development. Tail-formation is retarded or inhibited by all depressing conditions, such as low temperature, low concentrations of narcotics (Child '12), etc., but exactly the same conditions may increase head-frequency in the same pieces. The experimental data upon this point will be presented at another time.

IV. THE FACTORS WHICH DETERMINE LOCALIZATION OF THE NEW HEAD

In all longer pieces of *Planaria*, the head when it forms is localized at the anterior end of the piece. The basis for this localization is the axial gradient: how the gradient determines the localization we have now to consider. Attention has already been called to the fact that when a planarian (earthworm, etc.) is cut in two, the posterior piece is much more strongly stimulated than the anterior. The same relation is evident in contact stimulation. Slight stimulation of a given region produces much more marked effects posterior than anterior to it. In short, the whole mechanism of dynamic correlation in *Planaria* and similar forms is developed on the basis of the axial gradient. Correlation between regions of the body is chiefly in the posterior direction, anterior regions being relatively independent of posterior, and posterior regions relatively dependent upon anterior. That metabolic gradients exist in at least some nerves in the lower animals is known (Child '14 a) and there are reasons for be-

believing that conduction occurs more readily and to greater distances in the downward direction of the metabolic gradient. Conduction against the gradient is possible but requires a much stronger stimulus for a given distance. If the nerve impulse is a wave of chemical reaction, as seems now to be demonstrated (Tashiro '13), it is at least probable that in order to travel for any appreciable distance up the original gradient, the impulse must be strong enough to reverse the gradient temporarily as far as it proceeds, while a much weaker impulse may travel down the gradient for long distances. But whether this interpretation is correct or not, the fact remains that in planarians and many other lower forms, nervous and dynamic correlation (Child '11 a, p. 18) in general, is chiefly from more anterior to more posterior regions.

Admitting this fact, it follows that cells at the anterior end of a piece are in general more independent of other regions of the piece than the cells of any other level. It has been pointed out that the conditions necessary for head-formation are: first, embryonic cells of the species, second, physiological or physical isolation from correlative factors, and third, a sufficiently high rate of metabolism. It is evident that the condition of isolation from correlative factors is more completely fulfilled at the anterior end of the piece than elsewhere. At the posterior end, on the other hand, the new cells can never become physiologically isolated unless the axial gradient is eliminated, and in such cases we find that a head may actually arise at the posterior end of a piece. And in cases where the gradient is more or less permanently reversed, a tail may arise at the anterior end.

Briefly stated, the localization of new head and tail on a piece are due to the existence of the axial gradient. The head, which develops independently of other parts, develops at the anterior end because this is the only region of the piece where a sufficient degree of physiological isolation can possibly occur, as long as the axial gradient persists. The tail, which arises only as a subordinate part dependent upon and determined by more anterior regions, is localized at the posterior end of the piece because the cells in this region cannot become physiologically

isolated as long as the gradient persists, but develop under the dominance of more anterior parts and therefore produce a tail. By eliminating or reversing the gradient we may alter these localizations.

The headless piece represents a condition intermediate between the development of a head and of a tail at the anterior end. When rate y is sufficiently high in relation to rate x , to decrease the original gradient below a certain minimum in the anterior region of the piece, head-formation is simply inhibited, because neither x nor y dominates completely. But if rate y should become sufficiently high, as compared with rate x , to eliminate the old gradient and establish a new one sufficiently steep in the opposite direction in the anterior region of the piece, the region x will become a tail instead of a head. These points will be further discussed at another time on the basis of further experimental evidence.

V. THE FUNDAMENTAL REACTION SYSTEM OF THE SPECIES

Attention has already been briefly directed in other papers (Child '13 b, '13 c) to certain consequences for the problems of inheritance and development of the conception of the organism developed in these studies. If the head-region is a 'self-differentiating' system which arises in development independently of other parts and if other parts arise only in correlation with a head region or with a part which has already arisen in this way, we are forced to the conclusion that a single fundamental reaction system is the basis of both development and inheritance. The apical region or head region, or in animals which develop a morphologically differentiated nervous system, the cephalic region of the nervous system which is the dominant part of the head, is a closer approach than any other part of the organism to a morphological expression of this fundamental reaction system. In the lower animals, as well as in the plants, we see as a matter of fact that an isolated cell or group of cells capable of development produces an apical region or head except where it is prevented from doing so by correlation with already existing apical regions or heads.

The conclusion that the organism consists fundamentally of a single reaction system which is most closely represented morphologically by the apical region or head, or its dominant part, is forced upon us by the facts. Moreover, it is the only conception which enables us to account satisfactorily for the definite, orderly character of development, its progression in general from anterior to posterior regions, the dominance of the growing tip in plants and of the head in animals, and various other morphological and physiological characteristics of organisms. On the other hand, there are no real facts which indicate that the organism consists fundamentally of a multitude of independent determinants, factors or entities of any sort. Even the Mendelian phenomena do not demonstrate the existence of independent factors as entities, but merely indicate the existence of different capacities in a system, or of a variety of different systems. The localization of the visible realization of a capacity implies nothing as to the localization of the capacity. Any corpuscular theory of heredity and development demands the assumption of an anthropomorphic mechanism or 'vitalistic' principle of some sort for the management of the corpuscles. The conception of a fundamental reaction system as the basis of inheritance and development avoids all these as well as other difficulties; is developed from experimental data and brings into line a great number of facts which are very generally ignored by current theory.

The fundamental reaction system, dominance of the apical region and the axial gradient are all merely different aspects of the same general idea, viz., that the specific protoplasm of any organism consists fundamentally of a single physico-chemical reaction system which we may, if we desire, conceive as made up of a larger or smaller number of fundamentally similar partial systems. This system is the basis of inheritance and its dynamic capacities, the foundation of hereditary characters. The first step in organization and in embryonic development results from the establishment, in one way or another, of some region or portion of this protoplasmic reaction system as a region of higher

rate of dynamic activity. This region dominates development, becomes the apical or head region and determines the axial gradient or gradients, which constitute the dynamic basis of polarity and of individuation. The organization and development of various parts of the organism rests upon a similar basis of fundamental reaction system and dominance and subordination of parts resulting from differences in rate of reaction. Following papers will be devoted primarily to the development of this general conception on an experimental basis and its application to particular aspects of the problem of experimental reproduction, and secondarily, to the question of its significance for inheritance and development in general.

VI. SUMMARY

1. Whether or not a head shall arise at the anterior end of an isolated piece of *Planaria dorotocephala* is determined so fixedly during the first six or eight hours after section that head-formation cannot afterward be prevented by conditions which do prevent it when acting immediately after section. Head-determination undoubtedly begins immediately after section.

2. The period during which head-determination occurs is the period of stimulation following section, and in general the more a piece is stimulated by section the less likely it is to produce a head. Head-formation is a process opposed or antagonistic to the maintenance of the piece.

3. The development of a new head on a headless piece is not the restitution of a missing part but the first step in the development of a new individual. Whether a head shall develop or not depends primarily on whether the cells which give rise to new tissue at the anterior end of the piece become physiologically isolated to a sufficient degree to develop independently of other parts of the piece, or whether other parts prevent this development. In the former case a head arises, in the latter the piece remains headless.

4. If the group of cells which gives rise to the embryonic tissue at the anterior end of the piece is designated x , the similar group at the posterior end, z , and the remainder of the piece, y , we may express head-frequency in pieces in a very simple form, viz.: head-frequency = $\frac{\text{rate } x}{\text{rate } y}$. Tail-frequency, on the other hand, is directly proportional to rate y .

5. In pieces of considerable length the new head is localized at the anterior end of the piece because the axial gradient determines that the cells at this end are physiologically isolated to a much higher degree than the cells at the posterior end. A group of cells developing independently at a transverse cut surface gives rise to a head, but when developing in subordination to other parts, gives rise to a posterior end. In short pieces, biaxial heads, biaxial tails or reversal of polarity may occur according to the relations between the rates of the regions x , y and z .

6. The only logical conclusion from the data of experiment and observation is that a single fundamental reaction system is the basis of development and inheritance in each species, race or individual. The apical or head region, or the dominant part of that region, represents the fundamental reaction more nearly than any other part of the organism.

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TWO SEX-LINKED LETHAL FACTORS IN DROSOPHILA AND THEIR INFLUENCE ON THE SEX-RATIO

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SEVEN FIGURES

By a recessive lethal factor I mean any factor that brings about the death of the individual in which it occurs, provided its effect is not counteracted by the action of its normal allelomorph. The term is not intended to mean that some poison is produced that destroys the individual, but rather some defect is inherited that is serious enough to render the individual unable to live. The defect may be a physiological defect or a morphological malformation; in fact, a defect in any organ essential for the life of the larva, pupa, or imago would come under the general category of lethal. In the present case I have not attempted to discover where the elimination of the individual occurs, or to what specific defect it is due.

Lethal factors may be sex-linked or not, i.e., they may be carried by the sex chromosome or by an autosome. If the lethal factor is sex-linked it will kill any male in which it occurs, since the male has but one X chromosome. Such a factor can, therefore, never be transmitted through the male line, and, as a consequence, it is not possible to transfer a lethal factor from one lethal stock to another lethal stock, and in this way get two lethals together, because, as we have seen, all males that contain a sex-linked lethal factor die. If two sex-linked lethals should ever occur in the same female one must have arisen as a mutation independently of the other.

THE FIRST LETHAL FACTOR

The origin of this stock has already been described (Rawls,¹ Morgan,²). The first part of the data that follow includes those already given in my former paper. At the time when that paper was written the 'returns' were still coming in. Most of the counts here given for the first lethal were made by Mr. J. S. Dexter who was assisting me at the time.

Females from the lethal stock sent to me by Miss Rawls were mated to white-eyed males in pairs. Some pairs gave 1:1 ratios, i.e., equality in the sexes; and others gave 2:1 ratios, i.e., two daughters to one son. Those mothers that gave as many sons as daughters (e.g., pairs OM and K) were not heterozygous for lethal, and all their offspring should continue normal. Those mothers (e.g., pair L) which gave only half as many sons as daughters should be heterozygous for the lethal factor; and the missing sons should be those that received this lethal bearing sex-chromosome from their mother.

To bring out this difference clearly, daughters (heterozygous for white) from the normal lines OM and K were mated to white males. That they did not carry the lethal factor is shown in table 1 by the production of a 1:1:1:1 proportion, which is that normally expected from a cross between a white male and a red female heterozygous for white.

In contrast to the results of table 1 are those from the mating of daughters (heterozygous for white) from the pair L, which gave a 2:1 ratio, to white males. The results show that while half the daughters of pair L gave a normal sex-ratio (table 2), the other half gave the lethal sex-ratio of 2:1 (table 3).

The explanation of the fact that half of the daughters of L gave lethal sex ratios, and half gave normal ratios is that their mother was heterozygous for lethal. Half of her gametes were lethal and half non-lethal, so that of her daughters, half again should be heterozygous for lethal and half free from lethal.

¹ Biological Bulletin, vol. 24, 1913.

² Science, vol. 36, pages 718-20, 1912.

A comparison of tables 2 and 3 brings out the very striking fact that all the lethal sex ratios are characterized by the almost complete disappearance of a particular class of males—the red-eyed males, and that the absence or the smallness of this class is a *surer* index of lethal cultures than is the sex-ratio itself! Just as the red male class is almost exterminated, the white male class is almost undiminished, i.e., it is of about the size of each female class. That it is not nearer in size to the female class is due to the relatively poorer viability of these males.

This disproportionate effect of the lethal upon the size of the two male classes is due to association, i.e., the loci occupied respectively by the lethal and the white factors are very *close together* in the sex-chromosome. It would require a break of the chromosome between these two points to produce a red male which lives (non-lethal) or a white male which dies (lethal), and

TABLE 1

The offspring of twelve red-eyed daughters of O M, and of six red-eyed daughters of K when mated separately to white-eyed males

	RED ♀♀	WHITE ♀♀	RED ♂♂	WHITE ♂♂
O M 1.....	41	33	34	28
O M 2.....	21	21	13	20
O M 3.....	9	16	10	10
O M 4.....	30	29	44	34
O M 5.....	24	17	24	19
O M 6.....	13	11	14	11
O M 7.....	31	21	28	17
O M 8.....	24	25	22	26
O M 9.....	11	7	13	13
O M 10.....	12	8	8	8
O M 11.....	10	12	6	7
O M 12.....	11	16	16	23
K 1.....	45	37	54	41
K 2.....	38	38	36	38
K 3.....	39	37	34	35
K 4.....	55	33	46	56
K 5.....	42	40	45	44
K 6.....	8	13	9	15
Totals.....	464	414	456	445

TABLE 2

The offspring of red-eyed daughters of pair L, which when mated to white-eyed males, gave 1:1 sex-ratios

	RED ♀♀	WHITE ♀♀	RED ♂♂	WHITE ♂♂
L 1.....	32	47	34	32
L 2.....	36	38	30	31
L 3.....	13	25	18	24
Dup.* L 3.....	24	18	31	26
L 6.....	52	41	43	34
Dup. L 6.....	30	20	33	23
L 10.....	2	2	3	1
L 12.....	19	12	17	8
L 15.....	75	10	64	5
L 16.....	26	20	21	21
L 17.....	38	28	23	26
Dup. L 17.....	11	7	8	14
L 19.....	63	64	54	50
Dup. L 19.....	24	16	9	14
L 22.....	24	29	27	23
L 25.....	68	50	44	51
Dup. L 25.....	40	30	45	42
L 28.....	32	35	36	34
Dup. L 28.....	5	1	3	5
L 31.....	30	44	36	33
Dup. L 31.....	24	18	16	19
L 33.....	22	18	18	25
Totals.....	690	573	613	541

* The cases marked 'Dup.' refer to a second lot of offspring produced by the female of a given number.

it is assumed that breaking and crossing-over is infrequent in proportion as these loci are close together.

These relations can be represented by means of the following analysis and by figure A. Let X stand for the sex differentiator, l for the recessive lethal factor and L for its dominant allelomorph, w for the factor which determines white eyes and W for its normal allelomorph. The analysis for the cross of the female heterozygous for lethal and the white-eyed male is as follows:

P ₁	Lethal-bearing red-eyed ♀ X l W — X L W	
	Normal white-eyed ♂ X L w — —	
	F ₁ females	F ₁ males
	X l W X L w red ♀ (A)	X l W — red ♂ (dies)
	X L W X L w red ♀ (B)	X L W — red ♂ (lives)

TABLE 3

Red-eyed daughters of L, which when mated to white-eyed males, gave 2:1 sex-ratio.

	RED ♀♀	WHITE ♀♀	RED ♂♂	WHITE ♂♂
L 4.....	0	7	0	7
L 5.....	2	3	0	1
Dup. L 5.....	1	1	0	0
L 8.....	50	43	1	28
Dup. L 8.....	48	46	0	31
L 9.....	48	63	0	19
Dup. L 9.....	13	14	1	5
L 18.....	36	53	0	45
Dup. L 18.....	3	8	0	4
L 20.....	30	21	0	21
Dup. L 20.....	14	19	0	22
L 23.....	17	15	0	15
Dup. L 23.....	14	19	0	22
L 24.....	48	47	0	58
Dup. L 24.....	23	14	0	17
L 26.....	11	10	0	14
Dup. L 26.....	6	3	0	2
L 32.....	4	7	0	3
L 34.....	29	23	0	20
Dup. L 34.....	36	41	0	36
Totals.....	433	457	2	370

There are two classes of daughters (A and B) alike in appearance, but one of them (A) is heterozygous for lethal. The offspring of half the daughters (B) should be, as we have seen in table

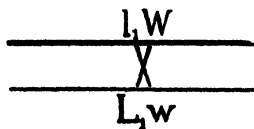


Figure A

2, non-lethal, and of the other half (A) lethal-bearing. Table 3 is the result of the mating of such daughters (A) heterozygous for lethal and white to white males.

In order to represent in a diagram what takes place in (A) let the pair of sex-chromosomes of the F_2 female be drawn in two.

parallel lines; the upper, heavier one, the maternal, and the lower, lighter, one the paternal chromosome. Lethal and non-white entered from the mother and non-lethal and white from the father. The sex-chromosomes of the F_1 female are represented with the loci for lethal and white close together. The cross between the lines indicates that crossing-over occurs between the loci in question. (The cross does not represent the postulated twisting, but only that it occurs in this general region.) Although for convenience the cross stands midway between the loci this is only a convention, for it will be understood that crossing over occurs at any point between the loci in question. The gametes of A will be of four classes, the first two, where no break has occurred between the loci L and W; and the second two, where a break has occurred, and crossing over has taken place. The first class of gametes (non-cross-overs) will be of two kinds, equally numerous, viz., $l W$ and $L w$. The second class of gametes (cross-overs) will also be of two kinds, viz., $l w$ and $L W$, but relatively few in number.

	$F_1 \text{ } \varnothing$		$X l W X L w$
	White σ^7		$X L w \sigma^7$
Gametes of F_1	$X l w - X l W - X L w - X L W$		
	$X L w -$		
	$F_2 \text{ females}$		$F_2 \text{ males}$
	$X l w X L w$ white	$X l w -$ white	(lives)
	$X l W X L w$ red	$X l W -$ red	(dies)
	$X L w X L w$ white	$X L w -$ white	(lives)
	$X L W X L w$ red	$X L W -$ red	(dies)

The analysis given above shows that rarely a red female should occur which is not heterozygous for lethal. The chance for her occurrence is the same as for the occurrence of the red male that lives, viz., 2:370 (table 3) or 1:185. Since therefore over 99 per cent of the red females are heterozygous for lethal we have kept the stock running by mating in mass cultures the red-eyed females to their white brothers. The presence of a female homozygous for non-lethal would make itself apparent at once by the appearance of red males which otherwise are extremely rare, and the reappearance of the 1:1:1:1 proportion of table 2. In this case the stock could be recovered by breeding

a few red females individually, and continuing one of the lines which gives a lethal sex-ratio and no red males or only an occasional one. Records were made of the offspring of pairs from some of these mass cultures of stock, and table 4, which is comparable with table 3, shows by the small number of red males that none of the females used were cross-overs (B) free from lethal.

TABLE 4

Red-eyed females (A) carrying the lethal factor gave the following offspring when mated in mass cultures to their white-eyed brothers

	RED ♀♀	WHITE ♀♀	RED ♂♂	WHITE ♂♂
L 242.....	118	126	2	93
L 181.....	36	43	0	30
L 201.....	180	138	2	125
L 8 L.....	294	243	0	207
L 9 G.....	38	36	0	23
L 9 K.....	18	27	0	4
L 2011.....	47	36	0	39
L 9 K A.....	97	89	0	68
Totals.....	828	738	4	589

If the analysis of this case is correct, the very small 'cross-over' class of red males should be entirely normal and should be unable to transmit the lethal characteristic. One of these males (of L 9, table 3) was tested by mating him to a wild female. There were produced 61 daughters and 48 sons, which is a fair approximation of the 1:1 ratio expected whether the father were lethal or not. But if the father were a male which carried the lethal, and by some means survived (i.e., if lethal were not invariably fatal in its action) then *all* these 61 daughters should be heterozygous for lethal and should give lethal ratios in F_2 . The F_2 generation was raised and consisted of 440 females and 377 males which is undoubtedly a non-lethal ratio.

Another of these red-eyed males (from L 242, table 4) was tested by mating to his red sisters heterozygous for lethal and white (type A). The F_1 consisted of 59 red females and 24 white males. The absence of red sons shows that the mother was of the type supposed, and the fact that there were twice as

many females as males shows that the male was non-lethal; for, had he been lethal, half the daughters of this cross would have been homozygous for lethal and would not have appeared. This would have resulted in equality of red females and white males. The test of this male was continued into F_2 by breeding three F_1 pairs, which gave:

	red ♀ ♀	white ♀ ♀	red ♂	white ♂
L 242 AA.....	25	0	12	0
L 242 AB.....	34	0	20	0
L 242 AC.....	12	11	7	19

This result is in conformity with the view that the grandfather was non-lethal, and impossible on the view that he was lethal. On the first view there should be two kinds of daughters (exclusive of cross-overs), viz., those heterozygous for lethal and free from white which would give a 2:1 sex-ratio, and those free from lethal but heterozygous for white (type B) which should give a 1:1:1:1 proportion. Fortunately both types were met with among the three daughters selected at random.

Theoretically, none of the white F_2 females in the cross analyzed should be heterozygous for lethal, except those rare cases due to crossing-over. These should be as frequent as the cross-over males, viz., one in about two hundred. Of the ten granddaughters tested, nine gave (table 5) 1:1 ratios, but one (L 9 D) gave apparently a 2:1 sex-ratio, although it was not seen in time to test further. Such a female mated to a red male in P_1 and the daughters again mated to red males should give results in many ways the converse of those obtained in tables 3 and 4, for, now, the red males would live and the white males die.



The locus of Lethal I

In table 3 the number of cross-overs (red males) was two, and of non-cross-overs (white males) was 370. Table 4 gives 4 cross-overs to 589 non-cross-overs. The total of 6 cross-overs to 959 non cross-overs gives the percentage of crossing-over between the loci lethal and white as 0.6 per cent.

TABLE 5

Offspring of white-eyed daughters of lethal flies mated in pairs to white-eyed males

	WHITE ♀♀	WHITE ♂♂
L 9 D.....	39	17
L 241.....	100	100
L 9 F.....	44	30
L 202.....	136	121
L 2421.....	64	56
L 2424.....	14	19
L 2425.....	37	30
L 2426.....	42	54
L 2427.....	32	30
L 2428.....	24	17
Totals.....	532	474

On the scheme of locating the factors that we have employed, 0.6 per cent of crossing-over means that lethal lies at a distance of 0.6 of a unit from white, but in which direction from white is not so far shown. In order to determine on which side of white the lethal lies, it is necessary to know the distance between lethal and another known point of the chromosome. The second point chosen was the factor for miniature wing. Two daughters (type A, from L 9) were mated to eosin miniature³ males and gave the following F₁ offspring (table 6).

TABLE 6

	RED LONG ♀♀	WHITE-EOSIN LONG ♀♀	RED LONG ♂♂	WHITE LONG ♂♂
L 93.....	9	7	0	6
L 94.....	30	28	0	21
Totals.....	39	35	0	27

The red-eyed, long-winged daughters (from table 6) were mated to their white-eyed, long-winged brothers with the following F₂ results (table 7).

³ Eosin is allelomorphous to white, so that any linkage results given by white or by eosin have the same value, and can be combined. The female which is white-eosin in composition is distinguishable from the pure eosin.

TABLE 7

	RED LONG ♀	WHITE-EOS LONG ♀	EOSIN MIN. ♂	RED LONG ♂	EOSIN LONG ♂	RED MIN. ♂
L 93 B.....	256	250	118	0	90	0
L 93 B.....	94	70	54	0	24	0
L 94 a.....	128	102	64	1	47	0
L 94 a.....	59	56	45	0	35	0
L 94 c.....	215	182	123	0	82	0
Totals.....	752	660	404	1	278	0

In the above F_2 results the proportions of the males give a measure of the amount of crossing-over between the lethal and the white loci. The non-cross-overs number $404 + 278$ or 682. The data from the males of tables 3 and 4 bear on this same point. The combined data give $2 + 4 + 1$ or 7 for the number of cross-overs in comparison with $370 + 589 + 682$ or 1641 for the number of non-cross-overs with respect to the interval L-W. This gives 0.4 as the percentage of crossing-over.

The data (table 7) of the cross in which miniature is involved give as the percentage of apparent crossing-over between lethal and miniature 41 which is somewhat higher than the value derived from our other data for this case.

Similarly the white-eosin daughters (cf. table 6) were mated to their white-eyed, long-winged brothers and gave:

	wh.-eos. long ♀	white long ♀	white long ♂	eosin min. ♂	white min. ♂	eosin long ♂
L 93 A.....	126	143	48	61	43	23
L 93 D.....	69	52	46	37	23	25
	195	195	94	98	66	53

The white-eosin daughters that gave the above results should rarely contain lethal (because of linkage) and the result 1:1 confirms the expectation. Two classes of males, viz., white miniature and eosin long are smaller than the other male classes because they are due to crossing-over between the white-eosin and miniature loci.

The most convincing evidence bearing upon the order of these factors in the linear series is that derived from a consideration

of the class whose occurrence would necessitate a double cross-over. A double cross-over class is always much less likely to be represented than any single cross-over class. Inspection of table 7 shows that the class red miniature is the only one not represented. The order of loci which would make the red miniature the double cross-over class is lethal, white, miniature (L W M). If lethal were not outside of the white to miniature section of the chromosome (that is, if the order of factors were white, lethal, miniature, W L M) the double cross-over male class would be the red, long class. The fact that this class is represented argues against the probability of white, lethal, miniature as the order of factors and points to lethal, white, miniature as the correct order.

A confirmation of lethal, white, miniature as the order of factors was obtained by using yellow and miniature as the fixed points instead of white and miniature. A large amount of data has shown that yellow is about 1.1 units from white on the side of white away from miniature. The data just given show that lethal lies at a distance of 0.4 units from white and probably on the same side from white as does yellow. Lethal should lie, therefore, between yellow and white at a distance of 0.7 from yellow and 0.4 from white.

Sturtevant made the following mating (not published). A gray, lethal long-winged female was mated to a yellow miniature male. They produced 169 gray red-eyed females and 64 gray white-eyed males. The female used was, as shown by the sex-ratio, heterozygous for lethal and for white. Of her daughters, half should be heterozygous for lethal and should not bear white, while the other half should be heterozygous for white, but should not bear lethal.

Six females of the latter type crossed individually to their white brothers gave non-lethal F_2 ratios in which white males and females occurred. Six other females gave the following lethal ratios in which no white appeared (table 8).

TABLE 8

GRAY LONG ♀	YELLOW MINIATURE ♂	GRAY MINIATURE ♂	YELLOW LONG ♂	GRAY LONG ♂
53	10	0	5	0
58	19	1	9	0
63	14	0	9	0
87	21	0	6	0
47	9	0	9	0
67	13	0	6	0
375	86	1	44	0

If lethal is between yellow and miniature the double cross-over class is gray long. The data of table 8 shows that this is the class not represented in the males, and the result is in harmony with the view that the order of factors is yellow, lethal, miniature. Furthermore, these data give us the distance of lethal from yellow $\frac{1}{1.1}$ or 0.7, which is the expectation if lethal lies between yellow and white and at a distance of 0.4 from white ($1.1 - 0.4 = 0.7$). Both experiments taken together show by the agreement in linkage, and in the absence of particular classes that the chromosomal line-up is yellow, lethal, white, miniature. This linkage of 0.4 is the strongest met with so far, corresponding to a 'gametic ratio' of about 250:1.

The reappearance of Lethal I

In 1914 a cross was made between yellow white normal females and gray red abnormal males. The cross gave in the F_1 generation yellow white males and abnormal females. No disturbance of the sex-ratio was noted, although it was not looked for specifically. Thirteen F_1 matings in pairs were made. Three of these pairs gave a sex-ratio of two females to one male.

females				males			
gray red	yel. white	gray white	yel. red	gray red	yel. red	gray white	yel. white
70	69	0	1	73	1	0	0
56	53	0	1	51	0	0	0
54	54	0	0	46	1	0	0
180	176	0	2	170	2	0	0

Furthermore, there were no yellow white males in the 2:1 ratios, although as many are expected as gray red, unless some disturbance is present. In fact, in the other ten pairs as seen below, the number of males in these two contrary classes correspond numerically (gray red, 649; yellow white, 621). The absence of the yellow white males aroused a suspicion that a sex-linked lethal was present, close to the yellow and the white factors.

<i>females</i>				<i>males</i>			
gray red	yel. white	gray white	yel. red	gray red	yel. red	gray white	yel. white
707	687	5	10	649	9	5	621

About a dozen of the yellow white females (from the lot giving 2:1 ratios) were bred to eosin males (stock). Only four pairs produced offspring. The records are as follows:

	white eosin ♀	yellow white ♂
B ₁	11	9
G ₁	25	5
G ₂	26	14
C ₁	36	21

The twelve pairs were afterwards transferred to one bottle and gave as a mass result:

White-eosin ♀⁴
101

Yellow white ♂
33

Matings (in pairs) were made between virgin females from B₁, G₁, G₂, C₁, and also some from the mass cultures with eosin males of stock. The results are given in table 9, where it will be seen that there are two kinds of females—some giving a 1:1 ratio (with the yellow white male class fully represented) and others giving a 2:1 ratio (with the yellow white male class entirely absent). Of the females tested 13 were non-lethal bearing, and 10 were lethal bearing. This is the expectation if the 2:1 ratio is due to a sex-linked lethal. If we return to the original series B₁, G₁, G₂, C₁ we find that only one daughter of B₁ was tested. She was non-lethal bearing. Probably B₁ was non-lethal as the

⁴ Also one mosaic.

ratio 11:9 indicates. The other three females, G_1 , G_2 , C_1 were lethal bearing as their ratios had indicated.

An examination of the male classes of the lethal lots shows that the lethal factor in question is the original lethal I that has reappeared, now linked to white instead of to red as originally. As a consequence the lethal males are now the white males, while before they were the red males. The following calculations will substantiate these statements.

The yellow white females, that carry the lethal, received from their mother a sex-chromosome carrying yellow (y), lethal (l_1), white (w) factors (upper line, fig. B); and from their father a

TABLE 9

	FEMALES		MALES			
	eosin	white-eosin compound	gray eosin	yellow eosin	gray white	yellow white
B_1	38	49	54	0	0	46
C_1	49	72	52	1	0	61
	64	51	61	0	1	83
	99	52	78	1	2	60
	91	90	87	1	0	78
G_1 a	43	46	45	1	0	47
G_1 b	60	45	40	0	0	45
G_1 c	68	58	62	0	0	72
G_1 d	63	62	75	1	2	56
G_2 a	64	58	54	0	0	39
G_2 b	40	41	47	0	0	50
G_2 c	87	77	72	0	2	60
G_2 d	98	110	85	1	1	73
Total	864	811	812	7	8	760
C_1 a	27	41	47	1	0	0
C_1 b	59	70	76	0	0	0
C_1 c	89	76	86	1	0	0
G_1 a	52	75	59	1	0	0
G_1 b	35	50	46	0	0	0
G_1 c	83	82	61	1	0	0
G_1 d	72	81	60	0	0	0
G_2 a	103	103	96	0	0	0
G_2 b	77	86	72	0	0	0
G_2 c	13	16	9	0	0	0
Total	660	738	668	4	0	0

sex-chromosome carrying the factors gray (Y) non-lethal (L_2) and eosin (w^e). The two non-cross-over classes, the four single

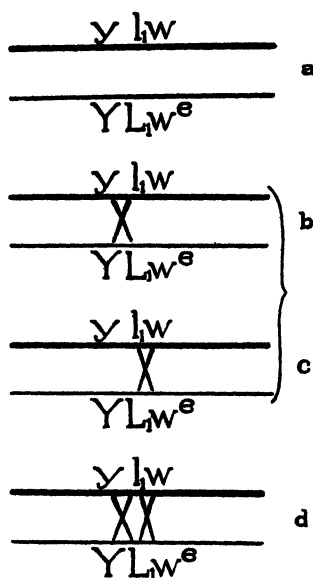


Figure B

cross-over classes, and the two double cross-over classes of gametes are as follows (see also fig. B).

Non-cross-overs.....	$\left\{ \begin{array}{l} y\ l\ w \text{ —(dies)} \\ Y\ L\ w^e \text{ —gray eosin} \\ y\ L\ w^e \text{ —yellow eosin} \\ Y\ l\ w \text{ —(dies)} \end{array} \right.$
Single cross-overs.....	$\left\{ \begin{array}{l} y\ l\ w^e \text{ —(dies)} \\ Y\ L\ w \text{ —gray white} \end{array} \right.$
Double cross-overs.....	$\left\{ \begin{array}{l} y\ L\ w \text{ —yellow white} \\ Y\ l\ w^e \text{ —(dies)} \end{array} \right.$

If we take the sum of all the male classes (668 + 4) and divide into the single cross-over class of flies (4) we get a percentage of 0.6 to represent the distance of the new lethal from yellow. This corresponds with the calculated percentage of the original lethal which is 0.7. In other words, this lethal lies near yellow and white—somewhat nearer to white. The single cross-over

between yellow and lethal occurs four times and the other cross-over, viz., that between lethal and white (to give gray white males) is not realized at all. The sons of the non-lethal sisters involve only two pairs of characters, hence there are two contrary single cross-over classes, viz., yellow eosin males and gray white males. These are expected in equal numbers as is practically realized (7YR and 8GW).

The data for non-lethals permits us to calculate again the distance of yellow from white (or eosin). The sum of all the male classes ($812 + 760 + 7 + 8 = 1587$) into the sum of the two cross-over classes ($7 + 8 = 15$) gives a percentage of .95 which is very close to the value (1.1) given by other experiments.

The question naturally suggests itself as to whether the reappearance of lethal I was due to mutation, or to descent from a common original stock. The stock used by Miss Rawls ('12) was wild stock that had been in the laboratory about a year when she found her high ratios running in it. Now the yellow stock, the white eyed stock, and the stock with abnormal abdomen had arisen before 1912, and had been maintained as pure stock. Hence the new lethal appears to have arisen as an independent mutation. Contamination is excluded on the grounds that it is well nigh inconceivable that a lethal could be inserted between yellow and white by a double cross-over, after contamination in the yellow white stock.

Summary of linkage data of Part I

From the preceding pages the data bearing on the linkage values have been collected and may here be summarized. In the following table for each linkage value the total number and the number of cross-overs is given; from which the percentage of crossing-over is calculated and is given in the last column:

	total	cross-overs	per cent cross-overs
Yellow lethal.....	1015	7	0.7
Lethal white.....	2559	7	0.27
Yellow white.....	5564	52	0.9
Yellow miniature.....	131	45	34.0
Lethal miniature.....	814	323	40.0
White miniature.....	994	397	40.0

THE SECOND LETHAL FACTOR

In an experiment with certain stock which had been inbred for three years, a pair produced:

73 females and 16 males (= 5:1)

These numbers represent the total output of this pair, or at least all the flies that were produced from one bottle. (A) Twenty-two of the seventy-eight females were mated to white miniature (of which two pairs produced nothing). (B) Twenty-nine of the seventy-eight females were mated to eosin vermilion males (of which six pairs produced nothing). (C) Twenty-four females of the seventy-eight were mated to eosin miniature males (of which four pairs produced nothing). The experiments with white and with eosin should give similar results, since white (*w*) and eosin (*w^e*) are allelomorphs.

The sixteen males were mated to eosin miniature, white miniature, and eosin vermilion females, but every one of these males proved sterile.

The output of the three lots of females mated to the respective males mentioned above is shown in A, B and C of table 10. Under each column, A, B, C, the progeny is provisionally classified, first, into those that give approximately, a 1:1 ratio, and then those that gave approximately a 2:1 ratio (or higher). A horizontal line separates these two classes.

The classification into these two groups may appear arbitrary, since the ratios in the two classes come very near to each other in some cases. For the present it will suffice to explain that the 2:1 ratio is expected if one lethal is present, the 1:1 ratio if no lethal is present.

From table 10 one can get no clue as to the location of the lethal factor that is assumed to be upsetting the normal sex-ratio, but by breeding the females to a male that carries the same sex-linked factors as their fathers, the location of the lethal factor is revealed.

Crosses with white miniature males

A number of daughters were taken from No. 12 (indicated by a star in table 10) and bred in pairs to white miniature males. Since No. 12 was carried further, I have selected it first for illustration.

The data are arranged in table 11, under two headings, viz., those pairs that gave a sex-ratio of 1:1, and those that gave 2:1. The data from pairs with a 1:1 ratio show nothing except the association of white and miniature. The data from the 2:1 ratio, in which the second lethal is present, show no males in the

TABLE 10

A				B				C			
NO.	FEMALES	MALES	SEX RATIO	NO.	FEMALES	MALES	SEX RATIO	NO.	FEMALES	MALES	SEX RATIO
2	34	51	.7	II	60	44	1.4	a	30	40	.8
3	66	51	1.3	VII	53	44	1.2	b	76	56	1.4
5	50	41	1.2	IX	7	8	.9	h	13	12	1.1
6	64	45	1.4	X	14	18	.8	j	54	53	1.
8	77	56	1.4	XI	63	64	1.	m	24	20	1.2
9	69	49	1.4	XII	58	56	1.	p	67	57	1.2
13	55	41	1.3	XIV	71	50	1.4	q	38	34	1.1
16	71	53	1.3	XV	24	29	.8	r	65	47	1.4
17	64	48	1.3	XVI	59	47	1.2				
18	78	63	1.2	XVIII	90	83	1.1.	c	58	35	1.7
				XIX	67	56	1.2	d	67	29	2.3
1	97	43	2.3	XX	44	38	1.3	e*	116	46	2.5
4	70	35	2.	XXI	57	52	1.1	f	124	22	5.4
7	72	24	3.	XXIII	50	40	1.2	g	68	21	3.2
10	59	38	1.5					i	53	19	2.8
11	47	25	1.9	I	79	37	2.1	k	62	30	2.
12*	122	32	3.8	III	114	48	2.4	l	80	31	2.8
14	64	26	2.5	IV	64	28	2.4	n*	58	20	2.9
15	78	34	2.3	V	74	31	2.5	o	65	28	2.3
19	86	38	2.3	VI	16	5	3.2	s	77	31	2.5
20	55	34	1.6	VIII	68	33	2.1	t	10	3	3.3
				XIII	53	26	2.				
				XVII*	96	30	3.2				
				XXII	21	14	1.5				

red-long class, which is the lethal class and, as will appear, also the double cross-over class.

One of the single cross-over classes is red miniature; the other, white long. Both are smaller than the non-cross-over class, white miniature.

TABLE 11
(From No. 12 of table 10)

NO.	RED LONG ♀	WHITE MIN. ♀	RED MIN. ♀	WHITE LONG ♀	RED LONG ♂	WHITE MIN. ♂	RED MIN. ♂	WHITE LONG ♂	SEX RATIO
1	43	24	13	6	28	31	8	20	1.
3	20	12	5	3	18	19	10	13	.7
7	48	31	7	10	7	26	9	4	2.1
8	37	37	18	15	23	39	23	9	1.2
9	33	26	13	8	22	27	15	8	1.1
13	33	34	10	14	22	21	11	13	1.4
15	25	18	13	16	18	23	13	6	1.2
18	27	26	14	16	38	33	13	20	.8
19	24	32	26	10	10	27	4	4	2.
21	39	32	19	18	12	35	19	6	1.5
22	13	25	5	6	24	6	9	13	1.
26	26	25	13	22	9	20	22	5	1.5
29	24	22	6	13	14	25	5	11	1.2
Total	392	344	162	163	245	332	159	132	
2	31	35	15	10	0	20	3	14	2.5
4*	28	24	16	16	0	19	3	7	2.9
5*	49	55	14	20	0	49	1	6	2.5
6	27	14	10	12	0	19	3	8	2.1
11	26	16	10	11	0	11	3	5	3.3
12	27	28	13	22	0	31	5	8	2.
14	23	28	5	9	0	29	3	9	1.6
16*	23	25	8	9	0	16	3	0	3.4
17	25	39	23	14	0	27	3	6	2.8
20	25	34	15	17	0	31	2	0	2.8
23	38	34	18	19	0	35	3	11	2.
24	34	24	14	10	0	31	5	7	1.9
25	21	31	13	10	0	19	2	2	3.3
27	25	27	5	8	0	17	3	4	2.7
28	26	23	12	8	0	23	3	5	2.2
30	29	27	20	17	0	33	5	6	2.1
31	31	20	11	14	0	26	4	6	2.1
Total	488	488	223	226	0	436	54	104	

In each generation the females that gave the highest sex-ratio were selected to breed from. Whatever disadvantage this method of selection may have, it at least makes more probable the retention of a 2:1 ratio.

TABLE 12

(From No. 16 of table 11)

NO.	RED LONG ♀	WHITE MIN. ♀	RED MIN. ♀	WHITE LONG ♀	RED LONG ♂	WHITE MIN. ♂	RED MIN. ♂	WHITE LONG ♂	SEX RATIO
a	46	25	10	24	0	30	7	10	2.2
b*	53	32	26	19	0	36	9	1	2.7
c*	47	49	22	19	0	26	6	4	3.8
d	37	34	17	11	0	29	3	5	2.7
e	35	37	25	19	0	29	2	4	3.3
f	31	30	10	15	0	20	2	2	3.6
g	55	49	20	28	0	66	9	14	1.7
h	43	51	25	15	0	40	6	1	2.8
i	20	20	9	9	0	14	4	4	2.6
Total	367	327	164	159	0	290	48	45	*

TABLE 13

(From No. b of table 12)

NO.	RED LONG ♀	WHITE MIN. ♀	RED MIN. ♀	WHITE LONG ♀	RED LONG ♂	WHITE MIN. ♂	RED MIN. ♂	WHITE LONG ♂	SEX RATIO
1	35	28	9	12	0	19	6	3	3.
2	35	39	10	18	0	36	2	11	2.1
3	39	32	24	19	0	29	2	4	3.1
4	30	37	18	10	0	31	6	1	3.1
5	36	44	21	6	0	29	4	0	2.2
6	36	36	16	8	0	38	5	1	2.2
7	44	26	22	14	0	35	3	10	3.
8	46	49	25	25	0	44	1	3	2.3
9	43	34	9	6	0	35	4	1	2.3
10	39	15	34	6	0	24	4	2	3.1
11	38	33	15	18	0	45	4	1	2.1
12	39	37	27	20	0	29	10	10	2.5
Total	467	410	230	162	0	394	51	47	2.6

From lot 16 of table 11 several virgin long-red females were mated in pairs to white miniature males. Their offspring are shown in table 12. Each pair shows in its offspring that the second lethal was present in the long winged red mother. A further generation was reared in pairs from red females of lots b and c of table 12. The offspring recorded in tables 13 and 14 show that the 2:1 sex-ratio continues.

Returning to table 11 there are records of two lots taken respectively from No. 4 and No. 5. The offspring from these pairs are here shown in tables 15 a and 15 b. Both show that lethal II is present as indicated by the absence (or rarity) of the red long males.

Before examining the preceding tables 10 to 15 in detail it may be helpful to have in mind certain theoretical relations. The location of factors for white (w) and for miniature (m) have been already determined. If we are dealing here with a lethal factor, it is essential to determine its position in relation to white and to miniature. For reasons that will appear later it is here assumed that this lethal lies between white and miniature (fig. C).

TABLE 14
(From No. c of table 12)

NO.	RED LONG ♀	WHITE MIN. ♀	RED MIN. ♀	WHITE LONG ♀	RED LONG ♂	WHITE MIN. ♂	RED MIN. ♂	WHITE LONG ♂	SEX RATIO
1	27	27	14	26	0	39	5	3	2.
2	26	35	6	15	0	23	7	5	2.3
3	53	59	30	32	0	56	7	2	2.7
4	61	49	39	13	0	44	7	3	3.
5	40	28	16	16	0	30	2	5	2.7
6	29	35	7	15	0	26	3	7	2.1
7	46	41	29	18	0	50	6	9	2.3
8	37	40	19	16	0	32	10	7	2.3
9	44	37	28	21	0	34	6	4	3.
11	59	55	27	25	0	67	8	7	2.2
12	16	10	1	21	0	23	3	1	1.8
13	41	34	11	11	0	31	5	3	2.5
Total	479	450	227	229	0	455	69	56	2.4

We are concerned with the heterozygous females only. Of the two sex chromosomes of these females, the one of maternal origin is indicated by the heavier line in the diagram (fig. C), the other, of paternal origin, is indicated by the lighter line in the figure. In the present case there entered from the maternal side the three factors: normal (red) eye (W), the second lethal (l_2), and normal wings (M); there entered from the paternal side the three factors: white eye (w), the normal allelomorph of lethal (L_2), and miniature wing (m).

In the case of this lethal the possibilities of interchange of factors between these chromosomes are shown in figure C, at *b*, *c*, *d*. If the crossing-over takes place between white and lethal, the case is indicated in (*b*). The two gametes that result from this single cross-over are $W L_2 m$ and $w l_2 M$; the latter never becomes realized in the male because he carries the lethal (l_2). Similarly, if the crossing over takes place between lethal and miniature the two gametes that are produced are $W l_2 m$ and $w L_2 M$, of which the former carries the lethal factor. There is another possibility, viz., the double crossing-over (*d*).

TABLE 15a
(From No. 4 of table 2)

NO.	RED LONG ♀	WHITE MIN. ♀	RED MIN. ♀	WHITE LONG ♀	RED LONG ♂	WHITE MIN. ♂	RED MIN. ♂	WHITE LONG ♂
a	26	22	14	12	0	23	3	6
b	23	19	11	13	1	12	3	8
c	34	39	21	18	0	28	10	8
d	30	24	12	16	2	15	5	9
Total	113	104	58	59	3	78	21	31

TABLE 15b
(From No. 5 of table 2)

NO.	RED LONG ♀	WHITE MIN. ♀	RED MIN. ♀	WHITE LONG ♀	RED LONG ♂	WHITE MIN. ♂	RED MIN. ♂	WHITE LONG ♂
b	16	16	6	8	0	11	1	1
c	34	30	8	8	0	26	5	6
Total	50	46	14	16	0	37	6	7

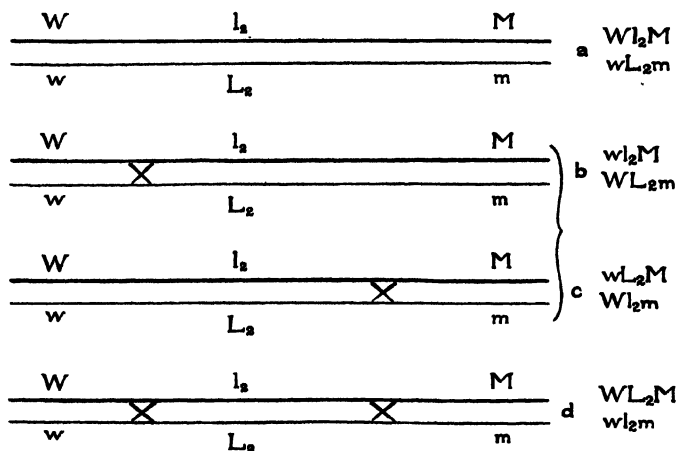


Figure C

In this case there is crossing over between L_2 and W and simultaneously between L_2 and M . Expressed in another way this means that the chromosomes cross between these two points, and the parts on the same side reconnect at the place of crossing. As a result of double crossing-over, the two gametes that result are $W L_2 M$ and $w l_2 m$, the latter of which never appears in the males.

If a female with these possibilities is crossed to a white miniature male (i.e., a male that carries these two recessive characters) the male classes will correspond to the gametic classes of that female except for those that carry the lethal which are not realized. The most frequent gametic class will be the non-cross-over class. The frequency of the other classes will depend on the distance of the factors involved from each other, because the chance of the crossing of the chromosomes taking place will be in proportion to the interval between the two loci. For this second lethal factor, therefore, the surviving classes of males are the following:

- Non-cross-over..... $w L_2 m$ —white miniature σ
- { Single cross-over..... $W L_2 m$ —red miniature σ
- { Single cross-over..... $w L_2 M$ —white long σ
- Double cross-over..... $W L_2 M$ —red long σ

Lethal and eosin vermilion

Twenty-nine females of the original 78♀♀ to 16♂♂ ratio were bred to eosin vermilion males and gave the ratios recorded in table 10 under I-XXIII. Some of these females from one culture No. xvii, with a ratio of 98:30, were again bred to eosin vermilion. Their offspring are shown in the record of pairs of table 16. Ten pairs gave a 1:1 ratio, and eleven a 2:1 ratio. As in the case of the cross with white eyes it is the red-eyed class of males, the lethal and also the double cross-over class which is deficient. (One record *jj* with three cross-overs may be due to contamination since the three occurred in the same bottle and in the same count).

TABLE 16
(From No. xvii of table 1)

NO.	RED ♀	EOSIN VERM. ♀	VERM ♀	EOSIN ♀	RED ♂	EOSIN VERM. ♂	VERM ♂	EOSIN ♂
		<i>No lethal.</i>		<i>Sex ratio</i>				
a	33	35	25	19	28	44	11	11
d	50	40	9	17	29	42	12	2
i	48	47	19	27	39	53	15	11
j	37	34	14	10	23	33	8	7
o	48	53	13	16	44	41	10	10
p	38	29	22	8	22	29	11	4
v	57	44	13	23	43	56	15	10
dd	34	31	12	12	22	39	15	11
ee	36	45	19	15	36	42	11	5
gg	47	49	22	19	35	41	12	7
Total	428	407	168	166	321	420	120	78
e	61	66	26	30	0	49	4	17
f	39	49	17	12	0	34	3	7
q	34	34	15	16	0	45	3	13
s	44	45	14	10	0	49	5	9
u	48	39	18	14	1	41	5	5
w	44	29	17	10	1	44	3	9
y	51	38	7	17	0	59	4	11
aa	47	39	6	11	0	47	5	13
hh	45	37	22	15	0	40	10	11
jj	58	37	13	34	3	29	2	12
ll	28	31	12	12	0	27	4	2
Total	499	425	167	181	5	464	48	109

From the lot *jj* (table 16) nine red-eyed females were bred to eosin vermilion males. The results show (in table 17) that the second lethal was present in the red-eyed females. In the lower part of the same table there are four cases recorded in which only vermilion and eosin vermilion offspring were produced. The sex-ratio 2:1 recurs. The results are undoubtedly due to four vermilion females (1, 5, 10, 14) from *jj* of table 10 having been taken instead of red females as intended.

From lot *c* of table 16 also seven red-eyed females were bred to eosin vermilion males. The results are given in table 18, which is similar to the preceding tables.

The sum of all the female classes that belong to the 2:1 division in tables 2 to 6 b and the sum of the corresponding males, is as follows:

		<i>females</i>			
red long	white min.	red min.	white long		
1964	1835	916	852	<i>males</i>	
3	1690	247	290		

The linkage shown in these results will be discussed later.

TABLE 17
(From No *jj* of table 16)

NO.	RED ♀	EOSIN VERM. ♀	VERM. ♀	EOSIN ♀	RED ♂	EOSIN VERM. ♂	VERM. ♂	EOSIN ♂
2	39	39	8	11	0	23	4	9
3	34	23	4	8	0	24	2	4
4	32	33	14	11	6	14	2	16
6	28	36	8	20	0	19	4	9
8	24	12	9	5	0	26	2	3
9	27	16	11	10	0	25	8	7
12	44	29	23	10	0	30	7	8
13	32	39	14	10	0	32	2	5
15	18	23	11	3	0	26	2	7
Total	278	250	102	88	0	219	33	68
1		40	36			39	1	
5		32	30			32	4	
10		39	41			24	8	
14		41	31			31	2	
Total		152	138			126	15	

TABLE 18
(From No. c of table 16)

NO.	RED ♀	ROBIN VERM. ♀	VERM. ♀	ROBIN ♀	RED ♂	ROBIN VERM. ♂	VERM. ♂	ROBIN ♂
1	47	25	19	14	0	34	5	10
2	53	42	13	9	0	38	8	5
3	40	35	18	17	0	28	8	11
4	48	28	13	16	1	36	7	13
6	28	31	13	10	0	32	4	2
8	19	14	16	6	0	26	3	4
9	29	36	15	12	0	25	8	5
Total	264	211	107	84	1	219	43	50

TABLE 19
(From No. e table 10)

NO.	RED LONG ♀	ROBIN MIN. ♀	RED MIN. ♀	ROBIN LONG ♀	RED LONG ♂	ROBIN MIN. ♂	RED MIN. ♂	ROBIN LONG ♂
6	35	29	16	14	31	28	11	7
8	24	22	12	14	22	19	6	10
9	19	24	12	11	25	26	10	15
10	28	20	10	12	31	24	12	11
14	22	15	10	6	16	23	11	10
16	20	12	9	9	27	15	12	14
17	11	11	9	4	8	12	12	1
18	24	29	12	12	20	16	17	9
19	33	21	8	11	18	26	14	14
21	22	22	8	12	25	23	8	8
24	45	38	17	21	28	23	19	13
26	12	13	6	4	5	8	6	8
27	18	26	13	10	19	22	12	7
Total	313	282	142	140	275	265	146	127
2	25	28	16	7	2	21	5	8
3	27	23	8	4	0	23	2	7
4	39	30	17	12	0	24	12	8
7	26	22	13	18	0	25	5	8
11	33	24	12	18	0	29	5	10
12	33	16	17	22	0	19	2	9
20	33	31	14	16	0	27	4	3
23	41	32	18	23	0	23	9	13
25	26	24	15	11	0	29	1	12
Total	283	230	130	131	2	220	45	78

Second lethal and eosin

The females of lot *e*, table 10, which gave a ratio of 116:46, were bred to eosin miniature males. As shown in table 19, fourteen of the females gave a 1:1 ratio, and nine a 2:1 (or higher) ratio. In the latter (2:1) class the double cross-over is the red long male. Two of these that appear must be due to a double cross-over. It is just possible, of course, that contamination may have occurred because these two males appeared in the same bottle and on the same day.

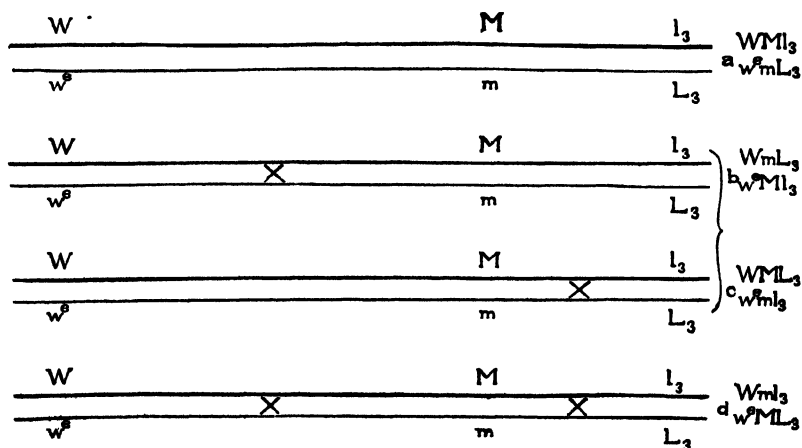


Figure D

The females of another lot, in table 10, viz., lot *n* with a ratio of 58:20, were also bred to miniature eosin males. The results shown in table 20 are strikingly different from any others so far obtained. The second lethal no longer appears to be present, yet in about half the cases the ratio is near 2:1. The question arises whether a new lethal has appeared, or whether we have in this set simply separated from the second lethal the other factor or factors that when combined with the second lethal gave the high 3:1 ratios.

No more light is thrown on this matter by the further history of this series. One lot only, viz., No. 39, seemed to give a 3:1

TABLE 20
(From No. n of table 10)

NO.	RED LONG ♀	ROBIN MIN. ♀	RED MIN. ♀	ROBIN LONG ♀	RED LONG ♂	ROBIN MIN. ♂	RED MIN. ♂	ROBIN LONG ♂	RED-ATO
1	56	39	32	31	46	53	32	12	1.1
2	39	31	14	12	22	21	25	6	1.4
3	45	37	30	25	27	41	28	12	1.3
4	71	76	30	30	41	42	20	23	1.6
5	56	56	28	33	16	20	20	11	2.6
6	56	50	34	19	24	24	18	10	2.1
7	54	42	30	25	26	32	20	3	1.9
8	44	28	30	31	40	39	23	27	0.95
9	43	40	19	21	24	27	13	18	1.5
10	42	34	28	27	22	25	25	5	1.8
11	54	39	32	29	19	36	19	13	1.8
12	56	41	35	26	63	39	23	24	1.0
13	61	52	30	33	34	52	27	9	1.4
14	61	67	36	35	39	63	25	19	1.4
15	43	51	25	39	35	25	24	12	1.5
16	45	36	31	20	36	25	28	8	1.4
17	59	65	26	28	55	61	26	29	1.0
18	48	24	31	19	29	28	21	12	1.5
19	72	61	29	26	31	29	40	12	1.7
20	37	36	25	22	14	23	18	5	2.0
21	46	28	26	27	21	26	25	13	1.4
22	51	48	33	24	31	30	20	9	1.7
23	51	33	26	20	28	29	21	16	1.4
24	40	37	32	19	40	24	19	6	1.5
26	56	45	28	28	22	27	18	12	2.0
27	65	45	30	33	31	35	31	18	1.5
28	48	50	28	28	21	37	24	8	1.7
29	50	25	16	10	27	13	13	9	1.6
30	33	33	25	12	11	38	17	5	1.6
31	38	34	30	15	21	24	23	6	1.4
32	32	42	17	22	19	35	15	10	1.5
33	38	32	22	31	20	25	18	5	1.8
34	30	41	13	18	28	17	18	21	1.2
35	29	32	13	18	30	21	13	7	1.3
36	39	20	9	9	24	20	9	8	1.3
37	38	30	30	20	20	35	17	10	1.5
38	27	23	22	9	13	10	7	2	2.5
39*	49	39	16	13	22	1	0	10	3.3
40	33	31	5	10	21	15	11	4	1.5
41	20	18	18	7	11	14	7	9	1.2
42	19	21	11	6	13	10	9	5	1.5
Total.....	1874	1612	1025	913	1117	1189	809	454	

TABLE 21
(From No. n of table 10)

no.	RED LONG ♀	EOSIN MIN. ♀	RED MIN. ♀	EOSIN LONG ♀	RED LONG ♂	EOSIN MIN. ♂	RED MIN. ♂	EOSIN LONG ♂	SEX RATIO
a	26	31	11	16	34	34	11	12	1:0.9
b	46	46	19	21	35	24	18	11	1:1.5
c	14	13	5	4	2	8	5	2	1:2.1
d	12	7	6	7	3	6	6	0	1:2.1
e	23	21	13	12	28	37	15	13	1:0.7
f	5	6	6	3	5	9	6	3	1:0.9
g	13	11	5	9	5	4	5	3	1:2.2
	139	135	65	72	112	120	66	44	

ratio and from this lot a few females were bred to eosin vermillion males. Their offspring are recorded in table 21. The data appear to verify the conclusion that the second lethal has gone while a disturbance of the sex-ratio remains. Can these results be interpreted to mean that the second lethal disappeared, and a third lethal, L_3 , that was present and gave the original high ratio of 78-16, has been retained?

If such a third lethal is here affecting the sex-ratio and if it lies beyond (to the right) of miniature as shown in figure F, then the deficient class is eosin long males. If the lethal kills then the only representative of that class will be double cross-overs and should be infrequent in proportion as L_3 is close to M. In fact, in a large number of cases in the table, the l_3 class, is behind the other male classes. In order, however, for the double cross-over class, eosin long, to be as frequent as appears in the table, the distance of L_3 to M would have to be very long indeed. In order to calculate this distance it would first be necessary to find out from table 20 those classes in which the factor occurs, but it is impossible to do this, for there are too many cases whose position is uncertain. If we make a single attempt to pick out such cases on the basis of the deficiency in the eosin long class we find that the most frequent class is that with a ratio of 1.5:1 and not 2:1 or more as would be the case if a lethal like the other two lethals is here present.

The meaning of the high sex-ratios that appeared in these cases must remain therefore unknown, and it is idle to speculate whether the results are or are not due to a non-sex-linked lethal, a sex-linked partial lethal, a sex-limited factor or factors, etc. The importance of following up this case was not appreciated at the time and this line was not continued except to breed seven individuals from No. 39⁵ (table 21).

Data from stock of Lethal II

The stock that carries Lethal II has been maintained by breeding long red females to white or to eosin miniature males from the regular eosin miniature stock. This has been continued to the present time. The results are shown in the next table, where,

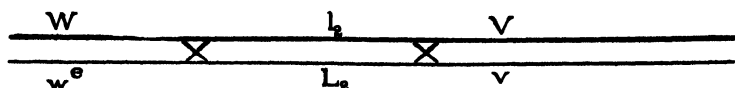


Figure E

through ten successive generations (bred in pairs), the inheritance of a 2:1 ratio has maintained itself. The horizontal lines in the first column separate generations; in some cases two or even three lines were tested, in each generation, in other cases only one. In all there are 13,749 flies recorded. The sex-ratio is 2.3:1. The order of the factors is shown in figure E. The percentage of cross-overs between white and lethal is 8.8, that between lethal and miniature is 16.3; and that between white and miniature is 24.7 which agree sufficiently with the percentages already given. The females are also available for cross-overs between white and miniature, and these give 28.3.

The linkage of Lethal II with white (or eosin) and with miniature

The diagram (fig. C) on page 103, will serve to recall the order in which the three pairs of factors enter. The F₁ female has received from her mother a sex-chromosome, bearing the factor for red (W) Lethal II (l₂) and long wings (M); and a sex-

⁵ No. 39 is peculiar in that eosin miniature and red miniature males are too few in numbers.

chromosome from her father, bearing the factors for eosin (w^o) normal or non-lethal (L_2) and miniature wings (m). The possible combination in the eggs of this F_1 female are repeated here:

Non-cross-over.....	$\left\{ \begin{array}{l} W l M \text{—red lethal long} \\ w^o L m \text{—eosin miniature} \\ W L m \text{—red miniature} \\ w^o l M \text{—eosin lethal long} \end{array} \right.$
Single cross-over	$\left\{ \begin{array}{l} W l m \text{—red lethal miniature} \\ w^o L M \text{—eosin long} \end{array} \right.$
Double cross-over.....	$\left\{ \begin{array}{l} W L M \text{—red long} \\ w^o l m \text{—eosin lethal miniature} \end{array} \right.$

TABLE 22
(Stock Lethal 11)

	RED LONG ♀	WH MIN. ♀	RED. MIN. ♀	WH. LONG ♀	RED LONG ♂	WH. MIN. ♂	RED MIN. ♂	WH. LONG ♂
A	428	381	161	125	0	362	31	21
B	365	337	166	175	1	276	45	60
C	509	502	229	232	0	368	65	74
D	316	329	95	115	0	305	26	63
E	318	289	104	88	1	278	31	62
F	312	259	95	81	0	259	19	61
G	73	105	24	27	0	94	3	26
H	124	104	68	52	1	105	14	36
I	118	114	50	46	1	117	16	33
J*	158	119	64	53	0	111	10	35
K	8	15	18	19	1	15	1	17
L	96	107	42	43	1	96	11	27
M	119	104	53	40	0	137	14	23
N	28	35	13	16	1	37	7	14
O	24	14	9	8	0	28	4	4
P	242	241	83	84	1	209	31	53
Q	271	302	114	116	1	338	31	63
	3509	3357	1388	1320	9	3135	359	672

* When Generation I was reached some of the red long daughters were bred to eosin miniature males (instead of using white miniature as before (A-I) under similar circumstances). Their offspring were as follows:

Red	long.	Wh. min.	Eos.	min.	Wh. long	Red	min.	Eos.	long
♀	♂	♂	♀	♂	♂	♀	♂	♀	♂
277	0	227	261	11	57	107	28	89	1

The long red females were again selected and bred to eosin miniature males to give generation J, K, L. Thus one generation is omitted in table 22, and from the summaries which include table 22.

Of the eight possible combinations, four carry lethal and will not be represented in the male classes of the next generation from the back-cross. The four surviving male classes represent:

- (1) A non-cross-over class (eosin miniature)
- (2) A single cross-over class (red miniature)
- (3) A single cross-over class (eosin long)
- (4) A double cross-over class (red long)

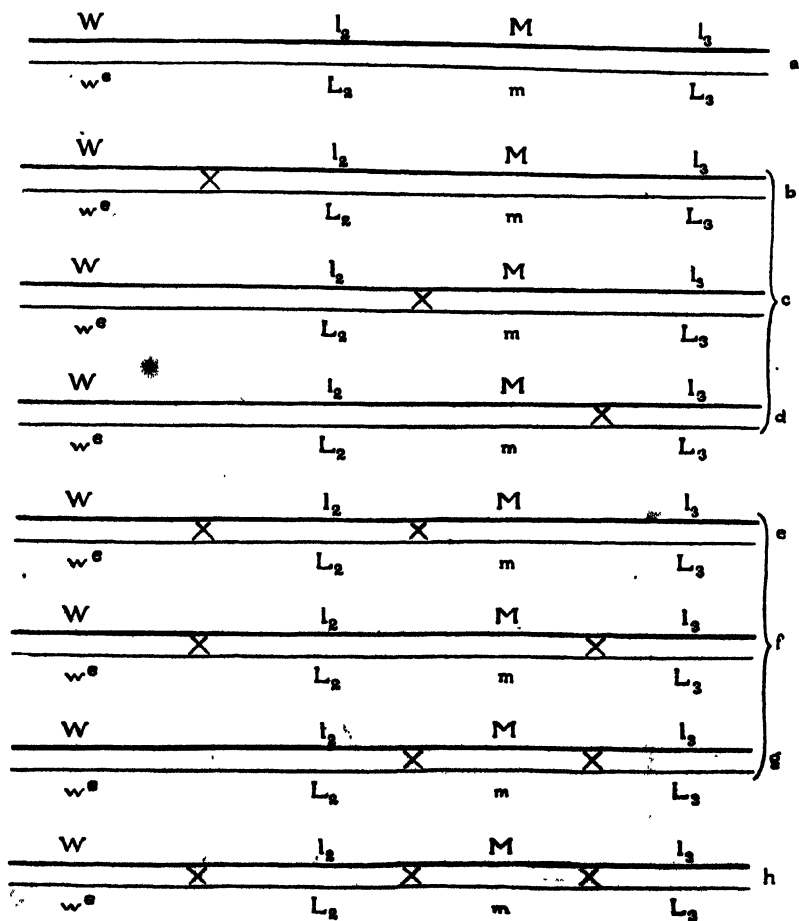


Figure F

TABLE 23

NO.	EOS. MIN.	RED MIN.	EOS. LONG	RED LONG	EOS. LETHAL	LETHAL MIN.	EOS. MIN.
XI	436	54	104	0	9.1	17.5	26.6
XII	290	48	45	0	12.5	11.7	24.2
XIII	394	51	47	0	10.4	9.6	20.0
XIV	455	69	56	0	15.2	12.3	27.5
XV ^a	77	21	31	3			
XV ^b	37	6	7	0			
XIX	220	45	77	2	13.7	22.4	35.5
XXII	3135	359	672	9	8.8	16.3	24.7
Total	4045	653	1039	14	9.9	15.6	25.1

In table 23 the data for the male classes available in the preceding tables are brought together in the first four columns. In the four columns that follow these, the percentages of crossing-over between: (1) eosin and lethal; (2) lethal and miniature; (3) eosin and miniature, are calculated for each of the experiments taken separately and for the sum total of the data.⁶

There is a considerable amount of difference in the percentages from the different experiments, as an examination of the table will show. If we take the totals as the more significant it appears that Lethal II lies nearly midway between eosin and miniature, but nearer to eosin. The total 'distance,' viz., 25 between eosin and miniature is smaller than that calculated from other data.

In the preceding experiments the females as well as the males give significant figures for eosin and miniature. In the next table, 24, these are brought together, and the percentage of crossing over between eosin and miniature calculated. This is 30.7 per cent.

In order to see whether the lethal factor has itself any influence on the results (which is not to be expected), the data from the sister individuals that gave a 1:1 ratio have also been utilized and are given in table 25. The percentage 'distance' is 32.4, which agrees with the calculation for the totals of the preceding table. Finally, the female classes alone are taken in the 2:1 count. These

⁶ The data in two cases, viz., 15a and 15b are not calculated separately, since the numbers are small.

give a percentage of 32.3. It seems to follow that the low percentages found when the males of the 2:1 ratio are taken alone is probably a disturbance due to viability in the males.

The linkage of Lethal II with eosin and with vermilion

As in the last case, the data for vermilion are to be considered. Figure C will serve to show the mode of entrance of the factors into the experiment if the factor V be used in place of M near

TABLE 24

NO.	SEX	RED LONG	WH. MIN.	RED MIN.	WH. LONG
XI	♀ 1:1	392	344	166	163
	♂ 1:1	245	332	159	132
	♀ 2:1	488	488	223	226
XII	♀ 2:1	367	327	164	159
XIII	♀ 2:1	467	410	230	162
XIV	♀ 2:1	479	450	227	229
XV ^a	♀ 2:1	113	104	58	59
XV ^b	♀ 2:1	50	46	14	16
XIX	♀ 1:1	313	282	142	140
	♂ 1:1	275	275	146	127
	♀ 2:1	283	230	130	131
XX [*]	♀ 1:1	1874	1612	1025	918
XXI	♀ 1:1	139	135	65	72
	♂ 1:1	112	120	66	44
XXII	♀ 2:1	3509	3357	1388	1320
Total		9106	8512	4103	3903

* The male class of XX is omitted because the white long males are far behind expectation.

TABLE 25

NO.	SEX	RED LONG	WH. MIN.	RED MIN.	WH. LONG
XI	♀	392	344	166	163
	♂	245	332	159	132
XIX	♀	313	282	142	140
	♂	275	275	146	127
	♀	139	135	65	72
XXI	♂	112	120	66	44
Total		1476	1488	744	678

which it really lies. The following list will serve to recall the possible combinations in the gametes of the F_1 female.

Non-cross-over.....	$\left\{ \begin{array}{l} W L_2 V \text{ —red lethal} \\ w^e L_2 v \text{ —eosin vermillion} \\ W L_2 v \text{ —vermillion} \\ w^e l_2 V \text{ —eosin lethal} \end{array} \right.$
Single cross-over.....	$\left\{ \begin{array}{l} W l_2 v \text{ —lethal vermillion} \\ w^e L_2 V \text{ —eosin} \end{array} \right.$
Double cross-overs	$\left\{ \begin{array}{l} W L_2 V \text{ —red} \\ w^e l_2 v \text{ —vermillion} \end{array} \right.$

In table 26 the available data are given in the first four columns, and the percentage distance between eosin and lethal, and lethal and vermillion, and eosin and vermillion in the following three columns. The calculations are given for the experiments taken singly, and for the totals. There is again a considerable discrepancy in the separate counts. The totals show that lethal lies between eosin and vermillion, but nearer to eosin. The total percentage distance between eosin and vermillion is 27.9.

As before, a calculation from the total data was made, table 27, on the basis of the male and female cases taken together, which

TABLE 26

NO.	EOS. VERM.	VERM.	EOSIN	RED	EOS. LETHAL	LETHAL VERM.	EOS. VERM.
XVI	464	48	109	5	8.5	18.2	25.1
XVII	219	33	68	0	10.3	21.2	31.5
XVIII	219	43	50	1	14.1	16.3	29.7
Total	902	124	227	6	10.4	18.5	27.9

TABLE 27

NO.	SEX	RED LONG	EOS. VERM.	VERM.	EOSIN
XVI	♀ 1:1	428	407	168	166
	♂ 1:1	321	420	120	78
	♀ 2:1	499	425	167	181
XVII	♀ 2:1	278	250	102	88
XVIII	♀ 2:1	264	211	107	84
Total		1790	1713	664	597

gives 26.5 per cent. Similarly the totals based on the experiments with 1:1 ratios gives 25.2; and those based on the females alone of the 2:1 ratios give 27.5. The difference in these is not greater than is expected. The lower percentage for the males taken alone is again probably due to viability.

Summary of linkage data of Part II

In the following table all the data available in the preceding pages (Lethal II) bearing on each linkage value have been brought together in a grand total:

	total	cross-overs	per cent cross-overs
White (or eosin) lethal II.....	8,152	812	10.0
Lethal II, miniature.....	6,407	974	15.2
Lethal II, vermilion.....	1,604	313	19.5
White (or eosin) miniature.....	36,022	11,048	30.7
Eosin (or white) vermilion.....	6,023	1,612	26.8

In the preceding table there are some discrepancies that appear when the percentages of crossing-over of the different counts of the same experiment are compared. Discrepancies like these may be due sometimes to insufficient numbers, sometimes to variations in differential viability (including the lagging behind of some of the male classes); in the case of lethals the absence of the 'contrary' classes (since one class dies) makes it impossible to check up viability in the male classes. Special external and internal conditions may at times affect the degree of interchange between the homologous chromosomes, which, by changing the gametic ratio, will affect the realized classes.

To determine the loci of factors accurately special studies and corrections are necessary. The present data pretend no more than to give the approximate positions of the loci, and, in this sense, the results are as consistent as is to be expected under the conditions of the experiments that were carried out with another end in view.

SEX-RATIOS HIGHER THAN 2:1

A glance at the last columns of the tables will show in many cases that the sex-ratio is higher than 2:1. Most of these differences are obviously chance deviations. There are some, however, that are so high as to suggest higher ratios. In fact, the start was made with a 5:1 ratio. It seemed at first as though those ratios higher than 2:1 might be due to two lethal factors that were simultaneously present, although this involves either two simultaneous mutations or the appearance of a new lethal in an egg that already contained one (through mutation or fertilization). Whether such an hypothesis is improbable will depend on how often lethal factors appear in stocks like these. Let us see how it might be shown that the high ratios could be explained as due to two lethals. If a third lethal l_3 were present it would be in all probability beyond miniature, or, like the second lethal, between miniature and white. On the first supposition the possibilities are shown in figure F. There are three single cross-over classes, three double cross-over classes and one triple cross-over. Of the sixteen possible classes of males only four would come through, as shown in the following list:

(a) Non-cross-over.....	$\left\{ \begin{array}{l} W \ l_2 \ M \ l_3 \text{ —not realized} \\ w^e \ L_2 \ m \ L_3 \text{ —eosin miniature} \end{array} \right.$
(b) Single cross-over.....	$\left\{ \begin{array}{l} W \ L_2 \ m \ L_3 \text{ —red miniature} \\ w^e \ l_2 \ M \ l_3 \text{ —not realized} \end{array} \right.$
(c) Single cross-over.....	$\left\{ \begin{array}{l} W \ l_2 \ m \ L_3 \text{ —not realized} \\ w^e \ L_2 \ M \ l_3 \text{ —not realized} \end{array} \right.$
(d) Single cross-over.....	$\left\{ \begin{array}{l} W \ l_2 \ M \ L_3 \text{ —not realized} \\ w^e \ L_2 \ m \ l_3 \text{ —not realized} \end{array} \right.$
(e) Double cross-over.....	$\left\{ \begin{array}{l} W \ L_2 \ M \ l_3 \text{ —not realized} \\ w^e \ l_2 \ m \ L_3 \text{ —not realized} \end{array} \right.$
(f) Double cross-over.....	$\left\{ \begin{array}{l} W \ L_2 \ m \ l_3 \text{ —not realized} \\ w^e \ l_2 \ M \ L_3 \text{ —not realized} \end{array} \right.$
(g) Double cross-over.....	$\left\{ \begin{array}{l} W \ l_2 \ m \ l_3 \text{ —not realized} \\ w^e \ L_2 \ M \ L_3 \text{ —eosin long} \end{array} \right.$
(h) Triple cross-over.....	$\left\{ \begin{array}{l} W \ L_2 \ M \ L_3 \text{ —red long} \\ w^e \ l_2 \ m \ l_3 \text{ —not realized} \end{array} \right.$

One of the four that are possible is the triple cross-over or red long. A triple cross-over is a very rare occurrence and would

not be expected within the numbers that appear in the experiment. The double cross-over is eosin long whose expectation is also rare. In table 11 there are two lots, Nos. 16 and 20, in which no males appear in this class, but as the expectation calls for very few flies here, it is unlikely that this can be the correct interpretation. If it were the correct interpretation we should get evidence of this third lethal when the red females of No. 16 were bred, as in table 12. But here there is no evidence of a third lethal, nor is there any in the following generations. It seems unlikely, therefore, that there can be any such lethal (i.e., one beyond miniature) involved in this experiment.

On the other hand, if the supposititious additional lethal should be between eosin and miniature, as shown in figure G, the effect would be to lower the class red miniature or eosin long below expectation for one lethal. In fact, the percentages of these classes are too low, but one would not be warranted, I think, from these data, in postulating a third lethal to account for this numerical discrepancy.

It seems probable, then, that the high ratios are either extreme cases of the 2:1 ratio, or that there is present some disturbing element not yet detected. The former view does not seem probable for the numbers are large. The second interpretation is more plausible, especially when the fact is recalled that the start was made from an exceptionally high ratio (5:1), and the line maintained by selecting the offspring of the pairs that showed the highest ratios.

There is another possibility that should at least be mentioned. The males hatch later than the females, and in the crowded bottles the males string out for some days after the females have ceased to hatch. In the present case there was some crowding, but the bottles were run to a finish, or practically so, in order to get the full male count. That the low male ratio is not due to this condition is shown by the fact that the non-cross-over class (eosin miniature males) compares favorably with the corresponding classes of females.

Sterility

An occasional test was made of the males that were obtained in the high sex-ratio pairs. Thus the sex-ratio from which table 1 came was 78♀♀ to 16♂♂ . Every one of these sixteen males was sterile. (Of their sisters only 13 out of 78 were sterile, or at least gave no offspring).

W	l_3	l_2	\bullet M	
w^e	L_3	L_2	m	a
W	l_3	l_2	M	b
w^e	L_3	L_2	m	
W	l_3	l_2	M	c
w^e	L_3	L_2	m	
W	l_3	l_2	M	d
w^e	L_3	L_2	m	
W	l_3	l_2	M	e
w^e	L_3	L_2	m	
W	l_3	l_2	M	f
w^e	L_3	L_2	m	
W	l_3	l_2	M	g
w^e	L_3	L_2	m	
W	l_3	l_2	M	h
w^e	L_3	L_2	m	

Figure G

In No. 39 (ratio of 115:33), seven out of fourteen females mated gave no offspring. One lot in table 1 shows a ratio of 116 ♀♀ to 46♂♂. Out of 31♀♀ tested, four produced nothing. Out of 14♂♂, seven were sterile.

One lot (xvii) of table 1 had a sex-ratio of 96♀♀ to 30♂♂. Out of thirty-nine females mated, only one gave no offspring. Six males tested were sterile.

No. 12 of table 1 had a sex-ratio of 122♀♀ to 32♂♂. Out of forty-three females mated, twelve produced nothing. Out of five males tested two produced nothing.

Summing up the results for the males, we get:

sex ratio	males tested	males sterile
78:16.....	16	16
116:46.....	14	7
96:30.....	6	6
122:30.....	5	2
Total.....	41	31

AN HYPOTHESIS TO ACCOUNT FOR THE EXCEPTIONALLY HIGH RATIOS

The following very high sex-ratios have been recorded for *Drosophila*:

	females	males
1	0	135
2	108	0
3	104	0
4	73	0
5	63	0
6	40	0
Quackenbush ⁷	43	0
7	33	0
8	31	0
9	68	0
1	52	1
2	30	0
3		

⁷ Quackenbush, L. S. Unusual broods of *Drosophila*. Science, N.S., vol. 32, p. 183, August 5, 1910.

	females	males	ratio
	312	3	104:1
Rawls ^a	169	5	34:1
	276	26	10:1
	291	54	5:1

In those cases where the offspring were tested (Rawls) it was found that the very high ratio disappeared. It seems to be due to a particular combination that was subsequently lost. If we assume that two linked lethals occurred, one in each of the sex chromosomes of the mothers that gave these high ratios, an explanation of the results is apparent. Such a female herself could live, since the lethals affecting different parts of the individual have each its normal allelomorph in the other sex chromosome. The eggs produced would be in the main of two kinds—half containing one lethal, half the other. Since all of the sons derive their single sex-chromosome from the mother, they will perish. But if crossing over in some of the eggs of the F_1 female should occur then in such eggs one of the chromosomes will get both lethals, and the other both the normal allelomorphs. The former type of egg, if fertilized by a male-producing sperm, would fail to produce a male; the latter type of egg would produce a normal male. The few males that appear may represent this class.

There are two ways in which the original double lethal female might be imagined to arise. A new sex-linked lethal might appear in one of the sperm-cells. If such a spermatozoon fertilizes an egg already containing a lethal a double lethal female that is viable would result. The frequency with which lethal mutants have turned up would make plausible this assumption. The same end would be reached if, in a female already containing one sex-linked lethal, another lethal should appear in the other sex-chromosome.

For two other results no hypothesis even can be suggested. First, the remarkable sterility found in the F_1 males when these high sex-ratios appeared; second, the converse case described by Quackenbush where 135 males and no females appeared.

^a Rawls, E. Sex ratios in *Drosophila ampelophila*. Biol. Bull., vol. 24, January, 1913.

I wish to acknowledge my indebtedness to Mr. A. H. Sturtevant and Mr. C. B. Bridges with whom I have discussed the theoretical questions involved in the experiments, and also my indebtedness to Miss E. M. Wallace and, to Mr. J. S. Dexter who have made the greater part of the 55,000 records that furnish the evidence on which the conclusions rest.

CLUSTER FORMATION OF SPERMATOOZOA CAUSED BY SPECIFIC SUBSTANCES FROM EGGS

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INTRODUCTION

In several papers F. Lillie¹ has described a very interesting specific phenomenon of apparent sperm agglutination which occurs when the sperm is mixed with sea water which has been in contact for a short time with a sufficient quantity of eggs of the same species (*Arbacia* and *Nereis*):

In the case of *Arbacia* the addition of two or three drops of egg-sea water $\frac{1}{4}$ (i.e., one volume of eggs to four volumes of sea water) which has stood half an hour, to about 2 cc. of fresh milky sperm suspension causes formation of agglutinations 1 to 2 mm. in diameter in a few seconds. The agglutination may be so strong that the fluid between the white agglutinated masses appears perfectly clear. The masses gradually fade from view in a few minutes, but microscopic agglutinations may remain half an hour or more.

The agglutination is, therefore, only transitory or reversible, as Lillie states. It is specific since e.g., the supernatant sea water of *Arbacia* eggs acts only on *Arbacia* sperm and not on other sperm.

It is very natural that Lillie should have been led to the idea that such a striking specific phenomenon as this agglutination must play a rôle in the process of fertilization and he has recently offered a very carefully worked out hypothesis which makes this phenomenon of agglutination not only the center of the process of fertilization and of artificial parthenogenesis but he even hints that it may be involved in the phenomena of heredity.

¹ Science, N.S., vol. 36, p. 527, 1912; Journ. Exper. Zool., vol. 14, p. 515, 1913.

Lillie's theory of the phenomenon of agglutination is an application of Ehrlich's side-chain theory, a fact which gives it additional interest.

In previous papers I have described the secretion of a substance by the ova of the sea urchin, *Arbacia*, in sea water, which causes agglutination of the sperm of the same species. The eggs of *Nereis* also secrete a substance having a similar effect upon its sperm. I therefore named these substances sperm-isoagglutinins. During the present summer I have ascertained that in the case of *Arbacia*, and presumably also of *Nereis*, the agglutinating substance is a necessary link in the fertilization process and that it acts in the manner of an amboceptor, having one side-chain for certain receptors in the sperm and another for certain receptors in the egg. As this substance represents, presumably, a new class of substances, analogous in some respects to cytolytins, and as the term agglutinin defines only its action on sperm suspensions, I have decided to name it fertilizin.²

The writer had for many years observed that when the eggs of the Californian sea urchin *Strongylocentrotus purpuratus*, were fertilized with sperm of their own species the spermatozoa would not always scatter but would form small clusters which were often visible with the naked eye. These clusters would disappear in a few minutes. The whole phenomenon resembles strikingly the phenomenon described by Lillie under the name of sperm agglutination, and is possibly identical with it.

The writer was interested to find out what the conditions of this cluster formation of the sperm and its relation to the process of fertilization were. Since he is not certain whether this cluster formation observed by him on the Californian sea urchin is identical with the observations of Lillie on the agglutination of sperm in *Arbacia*, he will confine himself to a discussion of his own experiments and observations, leaving it for future work to decide to what extent they harmonize with Lillie's observations and conclusions.

² Science, N.S., vol. 38, no. 980, p. 524, October 10, 1913.

METHOD OF OBSERVATION AND THE SPECIFIC CHARACTER
OF CLUSTER FORMATION

If we put one or more drops of a very thick sperm suspension of the Californian sea urchin, *S. purpuratus*, carefully into the center of a dish containing 3 cc. of ordinary sea water, and let the drops stand for one-half to one minute, and then by gentle agitation mix the sperm with the sea water, the at first rather viscous mass of thick sperm is in a few seconds distributed equally in sea water and the result is a homogeneous sperm suspension.

When the same experiment is made with the sea water which has been standing for a short time in a dish over a large mass of eggs of the same species, the result is entirely different. The thick drop of sperm seems to be less miscible and instead of a homogeneous suspension of sperm we get as a result the formation of a large number of distinct clusters which are visible to the naked eye and may possess a diameter of 1 or even 2 mm. The rest of the sea water is almost free from sperm. These clusters of spermatozoa last for from two to ten minutes and then dissolve by the gradual detachment of the spermatozoa from the periphery of the clusters. This phenomenon is to some extent specific. The sperm of the sea urchin *Strongylocentrotus purpuratus*, will give the cluster formation with the supernatant sea water of the eggs of *S. purpuratus*; the sperm of the sea urchin *S. franciscanus* will give the cluster formation with the supernatant sea water of eggs of its own kind as well as with the supernatant sea water of the eggs of *S. purpuratus*. In the latter case the clusters dissolve a little more quickly than if franciscanus sperm is added to the supernatant sea water of franciscanus eggs. The sperm of *purpuratus* will not form clusters with the supernatant sea water of the eggs of franciscanus. It is of interest that the specificity is not reciprocal in the case of these two sea urchins.

The sperm of neither formed clusters with the supernatant water of starfish eggs or of mollusc eggs.

The sperm of starfish (*Asterias ochracea* and *Asterina*) gave no cluster formation with the supernatant sea water of their own eggs or of the eggs of the two sea urchins.

We shall have to return to these data in a later chapter when we discuss the relation between cluster formation and fertilization.

The following experiments were carried on with the sperm of *S. purpuratus* and the supernatant sea water of the eggs of the same species, unless the contrary is stated.

APPARENT SURFACE TENSION PHENOMENA AND CLUSTER FORMATION

In analyzing the formation of these clusters the writer was struck with the fact that the cluster formation showed peculiarities which resembled the action of surface tension. The clusters were usually spherical, or had a tendency to become so. When two clusters were brought into contact with each other they fused at once into one spherical cluster with a larger radius, a behavior which would also be observed in the case of drops of substances immiscible with water under similar conditions. The formation of the clusters themselves resembled surface tension phenomena. When a drop of *purpuratus* sperm is gently agitated in a little dish with a few cubic centimeters of ordinary sea water streaks and cylindrical masses of sperm are formed in the water which, however, show nothing that reminds one of surface tension phenomena. The spermatozoa are gradually scattered without surface tension offering any resistance to the scattering.

If the same experiment is made in the supernatant sea water from the eggs—in egg-sea water—the streaks of sperm produced by agitation behave somewhat like cylinders of a very viscous substance which is immiscible with water, e.g., a viscous oil or a calcium soap. Short streaks or cylinders contract into spherical masses, the above described clusters; and long cylinders break up into a series of small clusters.

In an attempt to account for this apparent or real rôle of surface tension in cluster formation the writer thought first of the possibility that it might be due to an agglutination of the masses of sperm under the influence of the egg-sea water. A study of the real phenomenon of sperm agglutination, however, showed that it does not lead to any formation of spherical clusters. The writer had shown eleven years ago that real sperm agglutination

can be produced if we add 2 or 3 cc. of $\frac{N}{10}$ NaOH to 50 cc. of sea water.³ He found recently a good method of producing sperm agglutination with less alkali in the case of the sperm of starfish. When this sperm is put into 50 cc. sea water + 0.5 cc. $\frac{N}{10}$ NaOH it shows a tendency to agglutinate only after about one hour. But we can produce a real agglutination of the spermatozoa after about only twenty minutes when we put the sperm into the supernatant sea water of eggs or sperm of *purpuratus*. This agglutination is not specific, since it can also be produced by a great many other substances, e.g., cattle serum or even white of egg. In this case the spermatozoa at first stick together to form short rows or threads; and later the threads begin to stick together and form irregular networks. At no time is there any appearance of cluster formation or anything suggesting the phenomena of surface tension.

The writer is therefore under the impression that the cluster formation of the sperm in the supernatant sea water of its own eggs is a phenomenon of a different type from agglutination.

MOTILITY OF SPERM AND CLUSTER FORMATION

In observing the clusters the writer was struck with the fact that the spermatozoa at the periphery of a cluster are in free progressive motion, a fact which is incompatible with the assumption of agglutination. When the clusters were small or when the sperm suspension was thin it was possible to observe the spermatozoa which are in the center of the cluster. It was seen that the spermatozoa in the center also were in very lively motion, with the possible exception of small lumps or groups of spermatozoa which may have stuck together. The clusters reminded the writer of a dense swarm of insects which move like a coherent mass through space. These clusters move like one solid body through the water, notwithstanding the fact that the individual spermatozoa are free to scatter.

Under the influence of these observations the writer formed the idea that the cluster formation and possibly the apparent phenomena of surface tension might be the outcome of some tropistic

³ Loeb, Arch. f. d. ges. Physiol., Bd. 99, p. 323, 1903; Bd. 104, p. 325, 1904.

reaction of the spermatozoa. If this were the case, we should expect that anything that diminished the motility of the spermatozoa would lessen the tendency of the sperm to form clusters, and if the sperm were paralyzed completely the cluster formation would also cease completely.

It was easy to show that both assumptions were correct. To 3 cc. of a dense sperm suspension in ordinary sea water were added 1 or 2 drops of a 0.1 per cent solution of NaCN, and the whole thoroughly mixed. In one or two minutes the sperm lost its motility and did not regain it when put into sea water. When one or several drops of this immobilized sperm were added to the egg-sea water and when after one minute the dish was gently agitated, the sperm behaved exactly as if it had been put into normal sea water. Not a trace of cluster formation was noticeable; a slight agitation sufficed to bring about a perfectly homogeneous mixture of the sperm in sea water. After two hours the sperm became motile again when put into sea water. When such sperm, after the recovery of its motility, was put into egg-sea water a very powerful cluster formation occurred again.

These experiments were varied and always proved definitely that the whole phenomenon of cluster formation existed only when the sperm was motile.

There are other ways of paralyzing the spermatozoa. When the sperm of *purpuratus* is heated to a temperature of 35°C. or even 36°C. the sperm remains motile and the phenomenon of cluster formation is striking when a drop of such sperm is added to 3 cc. of egg-sea water and the mass is agitated. As soon as sperm is brought to a temperature of 37.6° or above and rapidly cooled, the motility is gone and no cluster formation takes place.

The same experiment was made with the sperm of *Strongylocentrotus franciscanus* and the supernatant sea water of eggs of the same species. When the sperm is heated to a temperature of 36.2° its motility continues and the cluster formation is not diminished. When the sperm is heated for one minute to a temperature of 37° the motility of the sperm is only diminished and only small clusters are formed. If the sperm is heated to 38° the motility of the sperm disappears and the phenomenon of cluster formation is impossible.

The same result is obtained if the motility of the sperm is diminished or annihilated through the addition of KCl to sea water.

These are all very striking demonstration experiments, which leave no doubt that the cluster formation and the apparent surface tension phenomena depend exclusively on the motility of the spermatozoa.

On the other hand, the writer convinced himself that in the true phenomena of sperm agglutination, the motility of the sperm is of no concern. We have mentioned the fact that the sperm of *Asterias*, when it has been in 50 cc. sea water + 0.5 cc. $\frac{N}{10}$ NaOH for fifteen or twenty minutes, undergoes a real agglutination when mixed with the supernatant sea water of different kind of eggs or of cattle serum. This real agglutination takes place just as well after the spermatozoa have been completely immobilized by KCN as before. We may therefore be sure that the cluster formation is not due to an agglutination.

CLUSTER FORMATION A POSSIBLE TROPISTIC REACTION

The writer's idea of a tropism underlying the cluster formation is at present only a mere working hypothesis about which it is therefore not necessary to say much. It is briefly this, that the spermatozoa which are rendered extremely active by the egg-sea water are at the same time repelled by it, in other words, that they possess a negative chemotropism or a negative differential sensibility towards the egg-sea water; while any small or large mass of spermatozoa at the boundary of or in egg-sea water acts as a center to which the isolated spermatozoa are positive. This would account for the fact that the cluster formation is a function of the motility of the spermatozoa and could also account for the apparent surface tension phenomena. We should also understand why the cluster formation (just like Lillie's 'agglutination') lasts only a few minutes. Since the egg-sea water must gradually diffuse into the mass of spermatozoa, the boundary at which they are repelled must finally cease to exist. As soon as the concentration of the active substance of the egg-sea water is the same or almost the same in the cluster or the mass of spermatozoa as in the surrounding sea water there is no more force active which may induce or preserve the cluster formation.

The idea of a negative reaction of the spermatozoa to the egg-sea water is in contradiction to Lillie's statement that the spermatozoa are positive to the egg-sea water. The writer is not quite sure whether Lillie's statement is based on a correct interpretation of his observations.

Lillie introduced a drop of Arbacia egg-sea water into a suspension of Arbacia sperm under a cover glass. In this case a dense ring of spermatozoa was formed "at the margin of the drop with a simultaneous formation of a clear external zone about 1.5 to 2 mm. wide; the ring then breaks up into small agglutinated masses and so becomes beaded" (p. 550). In the interior of the drop very few spermatozoa are found. If the spermatozoa were positively chemotropic to the egg-sea water, as Lillie suggests, they should rush into the drop instead of forming a ring around it. The writer is inclined to interpret this formation of a ring with a clear external zone around it as an indication that the spermatozoa are negatively chemotropic to the strong egg-sea water and possibly positively chemotropic to the more diluted egg-sea water or to the dense collection of spermatozoa in the ring. Those in touch with the margin of the drop are repelled by the drop and those at some distance from the drop are attracted towards the ring or towards the drop. This creates the dense ring next to the drop of egg-sea water and explains the formation of the clear space externally to the ring.

When a cluster scatters it does not scatter equally but one notices that isolated microscopic lumps or beads of spermatozoa may be left in the center of the original cluster. Later these beads or lumps scatter also. It is possible that the spermatozoa constituting these lumps or beads stick temporarily together and that this is caused by a specific substance contained in the egg-sea water. This agglutination, however, cannot account for the fact that cluster formation is only possible if the sperm is very motile. The cluster formation is, aside from the increased motility, the only striking phenomenon which the sperm of the Californian sea urchin shows in the presence of egg-sea water.

The writer wishes the statements of this paragraph to be taken only provisionally.

THE CONDITIONS WHICH DETERMINE THE SCATTERING OF
THE CLUSTER

The clusters (just like Lillie's 'agglutinations') have only a short duration of from two to ten minutes, as the circumstances may be. It was of interest to find out some of the conditions which determine their duration. It was found that the stability of the clusters depends upon the alkalinity of the sea water. In a neutral solution the big clusters may last a considerable time, half an hour or more, while in sea water to which a sufficient amount of alkali has been added the clusters may scatter in a minute. The reader must remember that if we add HCl or NaOH to sea water part of the added acid or base will be neutralized by the carbonates and phosphates of the sea water.

To 5 cc. supernatant sea water from *purpuratus* eggs were added 0, 1, 2, 3, 4 drops of $\frac{N}{10}$ NaOH and 3 drops of a dense suspension of *purpuratus* sperm were added to each. In all dishes a large cluster was formed. In the dishes with 4 and 3 drops of NaOH the clusters were dissolved almost instantly after formation, in the dish with 2 drops the resolution occurred more slowly and it lasted longest—about eight minutes—in the sea water to which no alkali was added.

In a second experiment to 5 cc. of the same egg-sea water 0, 1, 2, 3 and 4 drops $\frac{N}{10}$ HCl were added, and then *purpuratus* sperm introduced. ■ 5 cc. egg-sea water + 4 drops of HCl no cluster formation occurred, probably because the motility of the sperm was too rapidly annihilated. In the dish with 3 drops HCl only a trace of cluster formation was noticeable; with 2 drops a moderate cluster formation occurred and only in the two dishes with 0 and 1 drop of HCl was the cluster formation very powerful, since here the motility of the spermatozoa was not impaired. In the egg-sea water without acid the clusters disappeared much more quickly than in the sea water with 1 or 2 drops of acid.

Experiments in which neutral artificial sea water was substituted for normal sea water showed that at the point of neutrality the cluster formation is most durable. The big clusters continued to exist as long as half an hour, while in alkaline solutions they disappeared very rapidly. In acid solutions no cluster formation

was possible probably on account of the fact that the spermatozoa became immobile.

These and other experiments prove that an increase in the alkalinity of the solution shortens the duration of the clusters; in spite of the fact that an increase in alkalinity of the sea water favors the real agglutination of sperm.

The writer tried then to ascertain which salt solutions favor the formation of these clusters. To investigate this point the ovaries and testes of *purpuratus* were washed in an $m/2$ NaCl solution and then put directly into another $m/2$ NaCl solution without coming in contact with sea water. It was found that the supernatant solution of the eggs did never, or only exceptionally, give rise to cluster formation with the NaCl sea water; the reason for this may be partly the fact that the spermatozoa are practically inactive in a pure NaCl solution and that although the presence of the supernatant NaCl solution from the eggs stimulates the spermatozoa into activity this may not always be sufficient. The addition of KCl does not materially improve the cluster formation, the addition of the chlorides of Mg, Ca, Sr and Ba and of $MgSO_4$ vastly increases the cluster formation or induces it in an otherwise inefficient NaCl solution.

It is not possible to draw any conclusions from these facts upon the nature of the process underlying it.



THE ORIGIN OF THE SUBSTANCE CAUSING THE CLUSTER FORMATION

Lillie assumes that the substance which causes the phenomenon described by him as agglutination is given off by the egg itself though he states that the jelly which surrounds the egg—viz., the chorion—is saturated with this substance. The writer was curious to know whether the phenomenon of cluster formation depends upon a substance given off by the egg or whether it is due to a substance originating from the chorion. It could easily be shown that the latter is the case. Herbst had stated that the chorion of the sea urchin egg can be dissolved by acid. The writer therefore put a mass of eggs of *purpuratus* for three minutes

into 50 cc. sea water + 3 cc. $\frac{N}{10}$ HCl. The eggs were constantly squirted with a pipette to prevent them from sticking to the glass and were then transferred to normal sea water. They were then washed five times in succession in normal sea water under constant squirting with a pipette and then left standing in a refrigerator with a small volume of sea water. At no time did the sea water in which these eggs were kept give any trace of a cluster formation with fresh sperm. The supernatant sea water was tested a few hours after the acid treatment and two or three times daily on four consecutive days. These eggs which had apparently lost their chorion had permanently lost the power of giving off to the sea water a substance which causes the cluster formation of the spermatozoa of the same species. If the substance were constantly given off by the egg it should have been found after some time in the supernatant sea water. The experiment was repeated a number of times with the same negative result.

On the other hand, it was easy to show that the acid sea water (50 cc. sea water + 3 cc. $\frac{N}{10}$ HCl) in which the eggs had been washed contained the substance which is responsible for the cluster formation in large quantities. This acid sea water was filtered and the filtrate neutralized with NaOH (with neutral red as an indicator). The neutralized sea water gave with sperm of the same species a very powerful cluster formation.

This neutralized sea water kept the power of inducing cluster formation for about three days (during which time it stood in the refrigerator) but had lost it the fourth day.

This seems to indicate that the substance causing cluster formation is derived from the jelly-like substance surrounding the egg (the chorion) but does not emanate from the egg itself.

If this substance which causes the cluster formation should be identical with the substance which Lillie calls 'fertilizin,' which is very probable, it is obvious that his conclusion that the substance comes from the egg is untenable. This would also make it impossible to attribute to this substance a rôle in the process of artificial parthenogenesis.

CLUSTER FORMATION AND FERTILIZATION

Lillie's application of the side-chain theory to the problem of fertilization rests on the assumption that the substance which causes the phenomenon he describes as agglutination is indispensable for fertilization. This 'fertilizin' is in his theory an amboceptor which must combine at one end with the spermatozoön at another end with the egg; the 'fertilizin' when in combination with the spermatozoön undergoes a change and then fertilizes the egg. It is therefore a substance given off by the egg which in Lillie's opinion causes its fertilization, and not, as we all had hitherto assumed, one or more substances contained in the spermatozoön.

We have just seen that the substance which causes the cluster formation does not come from the egg but is given off by the chorion—or possibly is the chorion itself which is slowly soluble in sea water.

We can show in a number of different ways that eggs which have lost or do not possess the power of giving off a substance which induces cluster formation may possess the normal power of being fertilized. If we treat eggs of *purpuratus* for three minutes in 50 cc. sea water + 3 cc. $\frac{N}{10}$ HCl and wash them about five times in sea water they have lost the power of causing the cluster formation of the sperm of *purpuratus*. Such eggs can be fertilized immediately after the washing or at any time during the next two or three days if they are kept in the refrigerator. Their power of being fertilized is not in the least impaired. One hundred per cent of the eggs were invariably fertilized and the fertilization took place instantly after the addition of sperm. Practically all the eggs developed. The membrane was slightly abnormal which was an after-effect of the acid treatment. The power of the eggs of being fertilized remains unimpaired while their power of giving off substances which cause cluster formation is completely and permanently lost.

When we treat the eggs with a fatty acid instead of with a mineral acid they form, when transferred to normal sea water, a fertilization membrane. The fatty acid also dissolves the chorion and such eggs when washed afterwards lose their power of induc-

ing cluster formation of sperm. It is interesting that Lillie states that such eggs lost also their power of causing agglutination, which seems to suggest that Lillie's 'agglutination' and the writer's 'cluster formation' may be the same thing. Lillie states that such eggs which have formed a fertilization membrane have lost their power of being fertilized and he sees in this a support of his theory that without 'fertilizin' the egg can no longer be fertilized.

Eggs which have formed a fertilization membrane under the influence of butyric acid can easily be fertilized with sperm if the membrane is torn by shaking. The writer has repeated this experiment this winter and confirmed the earlier observations by Kupelwieser and himself to the same effect. He also made sure that the eggs which after the artificial membrane formation can be fertilized with sperm have completely lost the power of causing cluster formation.

We thus see that complete loss of the power of inducing cluster formation can be combined with maximal power of the eggs of being fertilized.

HYBRIDIZATION AND CLUSTER FORMATION

The best test for a possible connection between fertilization and cluster formation is afforded in the phenomena of hybridization. If the phenomenon of cluster formation were inseparably associated with the power of the eggs of being fertilized, we should expect that sperm should only be able to fertilize the eggs of a species if the egg-sea water of the same species caused the cluster formation of the sperm.

It is easy to show that no connection of this type exists. It is impossible to call forth cluster formation of the spermatozoa of the starfish *Asterias ochracea* with the egg-sea water of *purpuratus* and yet 100 per cent of the eggs of *purpuratus* can be fertilized with the sperm of *ochracea* and as many as 80 per cent of these eggs may develop. The writer showed that this hybridization takes place only in hyper-alkaline sea water and it was therefore necessary to test the possibility of cluster formation in both neutral and alkaline sea water; these tests gave always absolutely negative results.

The sperm of *purpuratus* shows no trace of cluster formation with the egg-sea water of *franciscanus* and yet the eggs of *franciscanus* are readily fertilized with the sperm of *purpuratus*.

If the cluster formation were caused by a substance which was necessary for fertilization in the sense of Lillie's theory these and probably many other hybridizations which occur should be impossible.

It often happens that in hybridization less than 100 per cent of the eggs are fertilized. The writer tried whether the yield of fertilized eggs could be increased if the egg-sea water from the species which furnishes the sperm be added to the mixture. This would furnish the sperm with the specific 'amboceptor.' It was found that hybridizations occur just as well if not better in normal sea water than if the egg-sea water from the species from which the sperm is taken be added.

All these facts contradict the assumption that the substance which induces the cluster formation of the spermatozoön is necessary for fertilization.

ARTIFICIAL PARTHENOGENESIS AND CLUSTER FORMATION

The writer has expressed the idea that the causation of the development of the egg either by a spermatozoön or by the agencies of artificial parthenogenesis is due to an alteration of the cortical layer of the egg which may or may not be accompanied by a membrane formation; and he has shown that all hemolytic substances are able to bring about this alteration. Lillie agrees with this idea but differs in regard to the origin of the agent which causes this change in the cortical layer of the egg in the case of fertilization by a spermatozoön. According to the writer, this change is caused by a substance contained in the spermatozoön while Lillie assumes that it is a substance contained in the egg which must, however, be activated by the spermatozoön in order to cause the alteration of the surface of the egg. It seems to the writer that Lillie's assumption is unnecessarily complicated. Moreover, if it should turn out that the substance which is responsible for the cluster formation is identical with the substance which Lillie calls 'fertilizin'—which is very likely the case—

Lillie's theory becomes untenable, since this substance does not, in all probability, originate from the egg but from the chorion and since there is, as we have seen, no connection between the presence of this substance and the power of the eggs of being fertilized.

A second difficulty which Lillie has not considered lies in the fact that the writer has shown that in addition to the membrane forming substance still another, namely a corrective agency, is necessary for the causation of the development of the egg. The cortical change induces development but the egg as a rule perishes if the second factor of artificial parthenogenesis is not applied (hypertonic solution or lack of oxygen). The writer is suspicious that even a third factor may be implied. It is under these circumstances difficult to see how the assumption that the 'fertilizin' causes development—leaving aside all other objections—can act as an adequate substitute for the known facts of artificial parthenogenesis.

Lillie sees a proof for his idea in the statement of Glaser⁴ that the filtrate from eggs of *Arbacia* ground up with an equal volume of sea water will cause normal unfertilized eggs of the same species to undergo one or more cell divisions if they are transferred from the filtrate after one or two hours to normal sea water. Lillie concludes from this that the egg contains its own fertilizing substance, the 'fertilizin,' and that it was this 'fertilizin,' liberated from the eggs when they were ground up and contained in the filtrate, which induced the cell division of the *Arbacia* eggs in Glaser's experiments. Waiving the question of how this 'fertilizin' was 'activated' without the presence of sperm, the writer sees no reason to assume that the egg extract acted through the 'fertilizin,' or any other specific substance, since he has shown that a large number of non-specific substances are able to induce the first cell divisions (without membrane formation) in the egg of *Arbacia*, e.g., traces of any weak base like HN₃, or protamine.⁵ A slight increase of osmotic pressure could also have such an effect. It even suffices to let the eggs of certain females lie for

⁴ Science, N.S., vol. 38, no. 978, p. 446, 1913.

⁵ Jour. Exp. Zool., vol. 13, p. 577, 1912; Arch. f. Entwicklungsmechn. d. Organ., Bd. 38, p. 409, 1914.

some time in sea water.⁶ The writer fails to see any reason for assuming that the cell division in Glaser's experiments was induced by 'fertilizin.' The fact that the composition of the mixture which he used was unknown is not sufficient proof that 'fertilizin' was the active agency.

In conclusion, the writer would like to call attention to the fact that the cluster formation described in this paper or the agglutination of sperm described by Lillie inhibit the fertilizing effect of the spermatozoa instead of enhancing it, since the cluster formation prevents the spermatozoa from reaching the egg. Even from a teleological viewpoint it is difficult to understand why a substance which only prevents the fertilizing action of the sperm should be a necessary link in such action. The writer is inclined to believe that the cluster formation or agglutination of sperm does not occur when fertilization takes place under natural conditions.

The writer is not quite sure whether his interpretation of the cluster formation of the spermatozoa as a tropistic reaction will have to be modified or not. If it should turn out to be essentially or partly correct, it might be worth while to point out that we have many examples of tropistic reactions which are of no possible use to the species, e.g., all the phenomena of galvanotropism which are only laboratory products.

The writer takes pleasure in expressing his thanks to Prof. S. S. Maxwell of the University of California for his kindness in putting the Herzstein Laboratory at New Monterey at the writer's disposal. *

SUMMARY

1. The writer describes the formation of clusters of spermatozoa which is observed when the sperm of a sea urchin is put into the supernatant sea water of eggs of the same species. This specific phenomenon of cluster formation may possibly be identical with the specific phenomenon described under the name of sperm agglutination in recent publications of F. Lillie.

⁶ Arch. f. Entwicklungsmechn. d. Organ., Bd. 36, p. 626, 1913.

2. The cluster formation resembles the phenomena of surface tension in various respects, e.g., inasmuch as the clusters are spherical or tend to assume a spherical shape, and inasmuch as the fusion of two clusters results in the formation of a larger spherical cluster. When sperm is put into ordinary sea water or the supernatant water of foreign eggs these apparent surface tension phenomena are not observed. In real sperm agglutination neither cluster formation nor the above-mentioned surface tension phenomena are noticeable.

3. It was found that the cluster formation is a direct function of the motility of the spermatozoa. As soon as the spermatozoa are immobilized by NaCN or by high temperature or by KCl the cluster formation ceases; as soon as the motility of the spermatozoa returns the cluster formation occurs again when the sperm is put into the supernatant sea water of eggs of the same species. The real agglutination of sperm occurs just as well when the sperm is immobilized as when it is motile. The cluster formation is therefore not a form of agglutination.

4. The clusters last only a few minutes, like Lillie's sperm 'agglutinations.' The writer has found that in a neutral solution they last much longer than in an alkaline solution and that they scatter the more rapidly the more the alkalinity of the sea water is raised by the addition of NaOH.

5. The writer offers tentatively a working hypothesis which assumes that the phenomenon of cluster formation is essentially or partly due to a negative chemotropism of the spermatozoa to the egg-sea water.

6. It is shown that eggs which have been treated with acid sea water lose permanently their power of producing a substance which causes the cluster formation of the spermatozoa of their own species; while the acid sea water in which the eggs were treated when filtered and neutralized with NaOH induces a very powerful cluster formation. If it is true that the acid dissolves the chorion (the jelly-like substance surrounding the egg) this experiment would prove that the substance which causes the cluster formation is not formed in the egg but in the chorion. If this substance is identical with the substance which Lillie calls

'fertilizin,' his theory concerning the rôle which this substance plays in the fertilization and development of the egg will meet with serious difficulties.

7. It is shown that eggs which have been treated with a mineral acid like HCl and which have permanently lost the power of causing a cluster formation of the spermatozoa can nevertheless all be fertilized with sperm of the same species and that the rapidity with which the sperm fertilizes these eggs is equal to that with which normal eggs are fertilized. When the acid used was a fatty acid and when membrane formation occurred the eggs also lost permanently their power of inducing cluster formation but retained their power of being fertilized by sperm, provided that the membrane was first torn.

8. The supernatant sea water of the eggs of *Strongylocentrotus franciscanus* will not induce cluster formation of the sperm of *Strongylocentrotus purpuratus*; yet the latter sperm fertilizes the eggs of *franciscanus*. The sperm of *Asterias ochracea* undergoes no cluster formation in the supernatant sea water of *Strongylocentrotus purpuratus*, no matter whether the sea water is normal or hyperalkaline although the starfish sperm readily fertilizes most or all the eggs of *Strongylocentrotus purpuratus* in hyperalkaline sea water.

9. The facts mentioned under paragraphs 7 and 8 prove that the substance which is responsible for the cluster formation is not necessary for the process of fertilization.

FERTILITY AND STERILITY IN DROSOPHILA AMPELOPHILA

I. STERILITY IN DROSOPHILA WITH ESPECIAL REFERENCE TO A DEFECT IN THE FEMALE AND ITS BEHAVIOR IN HEREDITY

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INTRODUCTION

The following series of papers embodies the results of my work on "Fertility and Sterility in *Drosophila ampelophila*." The first paper deals primarily with a case of sterility in which the female is affected, together with the effects of selection upon female sterility and its behavior in transmission. The second paper is concerned with the low fertility of a mutant, and the behavior of this low fertility in heredity. The third study deals with the effect on fertility of crossing different races. The fourth study treats of the effect on fertility of crossing within and without an inconstant race. The work has been in continual progress since the fall of 1911 when it was undertaken at the suggestion of Prof. T. H. Morgan.

Two investigators have studied sterility in *Drosophila*. Castle and his students found sterile individuals of both sexes in their

cultures. Moenkhaus found in his strains that sterility was practically confined to the males. He attempted to harmonize Castle's results with his own by the assumption that each had used a different measure of productiveness. Both investigators are in agreement that inbreeding can not be the cause of sterility, and that sterility is amenable to selection. From Castle's paper I gather that he considers low productivity and complete sterility in the female as related and that sterility in this case is related to egg structure. Moenkhaus seems to imply that sterility is due to some condition of the sperm. Evidence that bears on these questions is given in the following pages.

I wish to state in the beginning that my results warrant me in making a sharp distinction between fertility and sterility as it actually exists in the strains that I have used. Fertility, as I have found it, does not grade into complete sterility. There may however be gradations in fertility. The total sterility that appears in my cultures bears no relation to low fertility. Sterility as it affects the flies in my strains is due to a different condition from that operating to reduce the fertility. This statement is certainly true of the sterility as it affects the female. The distinction paves the way for bringing the results of other investigators under a common point of view. Failure to distinguish between sterility and fertility has I suspect led to confusion, certainly in the work of Castle, and probably in the results of Moenkhaus, because as I shall show the two things may relate to quite different phenomena. The inheritance of sterility and fertility in *Drosophila* must be separately treated if any progress is to be made.

In a culture of *Drosophila*, which I had been inbreeding and to which I shall refer as the inbred stock, there appeared an increasing number of sterile pairs. The sterility affected primarily the females as was evident by testing them with other individuals. In the sixth generation which had descended from a single pair of grandparents of the fourth generation, 51 pairs in a total of 105 pairs were sterile. There were 47 sterile females, 3 sterile males and 1 questionable case.

It seemed probable that the defect was due to something in the hereditary mechanism rather than to some factor in the environment, since the same defect reappeared among the grandchildren which hatched at different times during a period of two months. Since the effects of inbreeding were under observation I was at first inclined to attribute this sterility to inbreeding.

STERILITY AND INBREEDING

It is a popular belief that one of the effects of inbreeding is to induce sterility. Moenkhaus and Castle, however, after many generations of inbreeding with *Drosophila* could find no evidence in favor of such a view. Nevertheless, since sterility appeared in my 'inbred' culture to such a high degree it was tempting to attribute it to inbreeding. As Moenkhaus has clearly pointed out, if in a sterile inbred stock, the sterility can be eliminated by continued inbreeding, then inbreeding can not be held to be the causative agent. This is the test to which my own case has been put, and by selecting from those families that showed the sterility in the least degree a fertile race was produced. Tables 1 to 7 show the manner in which the sterility appeared in the inbred stock and its elimination by selection.

1. Sterility of the inbred stock

TABLE 1

Sterility of the first five inbred generations

GENERATION	NO. PAIRS TESTED	PAIRS STERILE	STERILE ♀	STERILE ♂
F ₁	16	1?		
F ₂	16	0		
F ₃	7	3		
F ₄	12	3		
F ₅ *	10	2	2	
Total.....	61	8	2	

* The ten pairs of F₅ were from a single pair of F₄.

TABLE 2

Sterility in the F₅ generation

ANCESTRY NO.	NO. PAIRS TESTED	NO. PAIRS STERILE	STERILE ♀	STERILE ♂
25	1	1	1	0
27	11	0	0	0
28	5	3	3	0
30	32	18	17	1
31	31	16	14	2
32	10	6	5	1?
	15*	7	7	0
Total.....	105	51	47	3+1?

* Miscellaneous pairs.

TABLE 3 *

Sterility in the F_7 generation

ANCESTRY NO.	NO.	NO. PAIRS TESTED	NO. PAIRS STERILE	STERILE ♀	STERILE ♂
27	134	17	7	4	
30	127	5	2		
32	129	12	6		
Total		34	15	4	

TABLE 4

Sterility in the F_8 generation

ANCESTRY NO.	NO.	NO. PAIRS TESTED	NO. PAIRS STERILE	STERILE ♀	STERILE ♂
127	149	26	11	10	1
127	151	21	5	5	0
127	160	16	3	3	0
129	164	4	2	2	0
127	165	8	3	3	0
127	166	4	2	1	1
127	167	3	1	0	1
134	172	28	12	10	2
Total		110	39	34	5

TABLE 5

Sterility in the F_9 generation

ANCESTRY NO.	NO.	NO. PAIRS TESTED	NO. PAIRS STERILE	STERILE ♀	STERILE ♂
172	228	28	6	5	1
151	226	8	0	0	0
151	227	4	0	0	0
Total		40	6	5	1

TABLE 6

Sterility in the F_{10} generation

ANCESTRY NO.	NO.	NO. PAIRS TESTED	NO. PAIRS STERILE	STERILE ♀	STERILE ♂
226	232	1	0	0	0
227	229	10	0	0	0
Total		11	0	0	0

TABLE 7

Sterility in the F_{11} generation

ANCESTRY NO.	NO.	NO. PAIRS TESTED	Nb. PAIRS STERILE	STERILE ♀	STERILE ♂
229	236	4	0	0	0
229	237	10	2	1	1
229	238	7	2	0	2
229	239	2	1	1	0
229	240	14	2	1	1
232	241	61	6	0	6
229	242	22	4	1	3
229	243	33	5	1	4
229	244	18	2	1	1
229	245	11	4	0	4
Total		182	28	6	22

Tables 1 to 7 show that in the beginning of the experiment only a few sterile pairs were found (table 1). Moreover, the fertile pairs were high producers (Part II, table 2) yet sterile individuals appeared in their offspring and these were largely females. At times fully half the females were affected, although there were undoubted cases in which the males were also affected.

It is to be noted in the eleventh generation, as shown in table 7, that out of 182 pairs only 6 sterile females appear. The sterility as it affected the female had been practically eliminated, for while it had been affecting 50 per cent of the females it affected at the end of the experiment less than 4 per cent. It might seem as though the character had been transferred to a certain extent to the male as 22 males out of 182 are recorded in table 7 as sterile. But the figures, as given here for the male, do not in all probability represent the actual facts in the case. Many of these males were very small, and after they had been paired for a few days practically all the males that proved sterile were crawling over the food with their wings drooping at their sides. This may or may not have prevented them from mating with the females. I was not able to follow the case further, but I have found no evidence in any of the other experiments that sterility, as it affects the females, can be shifted in heredity to the males.

That sterility is not due to inbreeding, and that selection is an effective agent in controlling it, is shown in the history of another strain, to which I shall refer as the truncate stock. This stock had been inbred for forty-two generations by Mr. Altenburg, a graduate student in the department. A great many sterile pairs were appearing in the strain and the broods were so small that it was somewhat difficult, as Altenburg told me, to keep the stock from dying out. Table 8 gives the history of the sterility as it appeared in this stock. When I took charge of the stock fully half of the pairs were sterile and yet on continued inbreeding a large percentage of the sterility disappeared. Table 11 shows that in F_4 , there were 3 sterile pairs in 21 pairs. That there were but few sterile individuals in the truncate stock at this time is also borne out by crosses that were made with fertile races. Sterility had been largely eliminated, and moreover this

had been brought about by selecting from those families that showed the least sterility (no. 17, table 9, and 17a, table 10).

It seemed to me at first that the large number of sterile individuals that appeared in this stock might bear a causal relation to the low productivity of the fertile pairs, but at the point in the experiment when the sterile individuals were eliminated there was no rise in productivity. I shall return to this question in the second part of the paper.

2. Sterility of the truncate stock

TABLE 8

Sterility of the truncate stock as it appeared in successive generations of inbreeding

	GENERATION											
	F ₃	F ₄	F ₅	F ₆	F ₇	F ₈	F ₉	F ₁₀	F ₁₁	F ₁₂	F ₁₃	F ₁₄
Number pairs tested.....	31	53	46	19	1	14	21	2	2	8	15	
Number pairs sterile.....	22*	27	12	6	0	2	3	0	0	1	2?	
Sex.....						2♂	1♂					

* Some doubtful cases are included.

TABLE 9

Sterility of the truncate stock in F₄₅ generation

	ANCESTRY NUMBER															TOTAL
	3	5	7	8	9	15	17	21	23	25	26	33	37	52	53	
No. pairs tested ...	1	1	1	4	2	1	8	4	3	4	4	4	5	3	1	46
No. pairs sterile ...	1	0	1	1	2	0	0	1	1	2	1	1	0	1	0	12

TABLE 10

Sterility of the truncate stock in F₄₅ generation

	NUMBER AND ANCESTRY							TOTAL
	5a	8a	17a	21a	23a	33a	52a	
Number pairs tested....	1	7	5	1	1	2	2	19
Number pairs sterile....	1	3	0	1	0	0	1	6

For the forty-seventh generation one pair was chosen from 17a. It is to be noted that no sterile individuals had appeared in the

TABLE 11

Sterility of the truncate stock as it appeared in the F_{4s} generation

ANCESTRY NO.	NO. PAIRS TESTED	NO. PAIRS STERILE	STERILE ♀	STERILE ♂
26	3	2	0	2
33	1	0	0	0
35	16	1	1	0
39	1	0	0	0
Total.....	21	3	1	2

last two generations of this family. Fourteen pairs were chosen from the F_{4s} generation, two of which were sterile. From this generation pairs were made up as shown in table 11.

It should be recalled that the truncate stock was bred at the same time and under the same conditions as the inbred stock, and while the former developed into a fertile stock the latter developed into a sterile female stock.

Attention is called here to the fact that the individuals in table 2 are the grandchildren from a single pair of the F₄ generation. Sterility appears in the different families and affects about 50 per cent of the females. Family 27 is an exception in that 11 pairs show no sterility although descended from the same grandparents. This small amount of evidence is in accord with the assumption that some individuals come through without the defect in their germ-plasm, and shows how selection has brought about its results. That one of the grandchildren in this combination had some factor which prevented the appearance in her of the defect is shown by the fact that the defect reappears among her daughters (table 3, no. 134). These numbers are too small to base a safe conclusion upon; but attention is called to them here, since the assumption is borne out in the experiments which follow.

BEHAVIOR OF STERILITY IN HEREDITY

In a study of sterility one meets at the outset a peculiar difficulty in that he cannot breed the animals that show the very defect which he wishes to study. Again there is no way by which sterile animals can be recognized except by pairing them with different mates which involves an additional amount of labor. Moreover

the same strain on inbreeding may behave differently in successive generations as has been shown in the history of the inbred and truncate stocks. An additional difficulty is met in finding a highly fertile strain against which to test a known sterile strain. In the face of these difficulties a number of detailed experiments have been required in order to determine (1) the method of transmission of the female sterility, and (2) the relation between low production and sterility in the truncate stock. I shall present first the evidence that bears directly on the method of transmission of female sterility.

TABLE 12
Sterility as it appeared in the Woods Hole stock

LOT	DATE	NO. PAIRS TESTED	NO. PAIRS STERILE	STERILE ♀	STERILE ♂
A	March 2, 1912.....	58	1	0	1
B	June 24, 1912.....	70	4	2	2
C	July 10, 1912.....	63	3	1?	2

A wild stock to which I shall refer as the Woods Hole stock was characterized by the presence of but few sterile individuals. Consequently this was good material against which to test the sterility of the inbred stock. An entirely fertile stock would be the ideal one to use but it is the experience of all who have bred these flies that a sterile pair is occasionally met without any assignable cause. Table 12 gives the history of the fertile Woods Hole stock.

At the time the following cross was made there appeared in the Woods Hole stock that was used as a control by placing under similar conditions, 4 out of 70 pairs that were sterile. There were 2 sterile males and 2 sterile females (table 12, Lot B). In order to determine the behavior of the sterility in the F_1 and F_2 generations, the inbred females were paired with the Woods Hole males. The reciprocal cross was also made. The offspring that resulted from this cross were paired together, brothers and sisters, for the F_1 generation. The grandchildren were paired for the F_2 generation. The offspring from three families of the inbred

stock were chosen to cross into the Woods Hole stock (see table 4, Nos. 149, 151, and 172). The pairs that were tested from these different families as a control show the sterility to have been present in different intensities in these families; No. 149, 11 pairs in 26 were sterile; No. 151, 5 pairs in 21 were sterile; No. 172, 12 pairs in 28 were sterile. That the controls give a fair measure of the sterility is shown by the crosses made into the fertile Woods Hole stock. These crosses are given, together with the sterility as it appeared in the F_1 and F_2 generations, in table 13 a to 13 f.

In these tables the first number gives the serial number of the cross (e.g., inbred male by Woods Hole female) and is designated as P_1 . The second column gives the number of pairs of offspring that were tested from the given family, for the F_1 generation, while the next column gives the corresponding number that were sterile. The fourth column indicates the number of pairs selected to become the parents of the F_2 generation. The succeeding columns give the corresponding number of pairs tested and sterility as it affected the different sexes.

Crosses between inbred ♂ and Woods Hole ♀

TABLE 13 a

Crosses between the inbred stock and the Woods Hole stock showing the behavior of the sterility in the F_1 and F_2 generations. Inbred ♂ No. 149 × Woods Hole ♀

P_1 NO.	PAIRS F_1 TESTED	PAIRS F_1 STERILE	ANCESTRY NO.	PAIRS F_2 TESTED	PAIRS F_2 STERILE	STERILE ♀	STERILE ♂
1	7	1	1a	12	1	0	1
			1b	10	2	2	0
			2a	13	3	3	0
2	7	0	2b	7	1	0	1
			2c	8	4	1	
			3a	12	0	0	0
3	5	0	3b	20	1	0	1
			4a	26	9	9	0
			4b	16	10	9	1
4	2	0	5a	10	1	1	0
			5b	20	1	1	0
			5c	12	0	0	0
5	5	1?					
Total.....	26	2		166	33	26	4

TABLE 13 b

Inbred ♂ No. 172 × Woods Hole ♀

P ₁ NO.	PAIRS F ₁ TESTED	PAIRS F ₁ STERILE	ANCESTRY NO.	PAIRS F ₂ TESTED	PAIRS F ₂ STERILE	STERILE ♀	STERILE ♂
6	8	0	6a	20	0	0	0
			6b	14	4	4	0
			6c	13	0	0	0
			6d	32	0	0	0
			6e	21	2?		
7	2	1♂	7a	4	1	1	0
			7b	28	3?		
			7c	20	1?		
8	5	0	8a	9	4	4	0
9	4	0	9a	16	2	2	0
10	3	1♀	10a	18	9	9	0
11	3	0					
12	6	2♂	12a	19	2	1	1
			12b	2	0	0	0
13	9	0	13a	16	2	2	0
			13b	15	1	0	1
14	6	0	14a	2	0	0	0
			14b	3	0	0	0
15*							
16*							
17*							
Total	46	4		252	31	23	2

* Sterile

TABLE 13 c

Inbred ♂ No. 151 × Woods Hole ♀

P ₁ NO.	PAIRS TESTED F ₁	PAIRS STERILE F ₁	ANCESTRY NO.	PAIRS TESTED F ₂	PAIRS STERILE F ₂	STERILE ♀	STERILE ♂
18	12	0					
19	17	0	19a	8	0	0	0
20	8	0	20a	21	0	0	0
21	8	0	21a	28	1?		
22	9	0	22a	25	2	1	1?
23	11	0	23a	15	0	0	0
24	12	1♂	24a	5	0	0	0
25	8	0	25a	10	1	1	0
26	13	0	26a	21	3?	3?	
27	9	0	27a	18	0	0	0
28	6	0	28a	20	2	1	
			28b	12	1	0	1
29	0						
Total	113	1		183	10	6	2

TABLE 13 d

Inbred ♀ No. 149 × Woods Hole ♂; reciprocal of 13 a

P ₁ NO.	PAIRS TESTED F ₁	PAIRS STERILE F ₁	ANCESTRY NO.	PAIRS TESTED F ₂	PAIRS STERILE F ₂	STERILE ♀	STERILE ♂
30	6	0	30a	50	4	2	2
31	7	0	31a	4	1?		
32	3	0	32a	20	3	3	0
33	5	0	33a	18	9	9	0
			33b	14	1	1	0
			33c	15	3	3	0
34*							
35*							
36*							
37*							
Total.....	21	0		121	21	18	2

* Sterile

Reciprocal: Crosses between the inbred ♀ and the Woods Hole ♂

TABLE 13 e

Inbred ♀ No. 172 × Woods Hole ♂; reciprocal of 13 b

P ₁ NO.	PAIRS TESTED F ₁	PAIRS STERILE F ₁	ANCESTRY NO.	PAIRS TESTED F ₂	PAIRS STERILE F ₂	STERILE ♀	STERILE ♂
38	10	0	38a	43	8	3	1
			38b	5	0	0	0
			38c	57	23	22	0
39	7	1♂	39a	15	6	4	
40	8	0	40a	15	1?		
			40b	17	6	6	0
41	1	0	41a	10	0	0	0
42	5	1♂+1♀	42a	24	0	0	0
			42b	35	20	17	1
43	8	0	43a	25	3	3	0
44	2	0	44a	40	5	5	0
45	2	0	45a	16	4	4	0
46*							
47*							
48*							
49*							
50*							
51*							
52*							
53*							
Total.....	43	3		302	76	64	2

* Sterile

TABLE 13 f

Inbred ♀ No. 151 × Woods Hole ♂; reciprocal of 13 c

NO. P ₁	PAIRS TESTED F ₁	PAIRS STERILE F ₁	ANCESTRY NO.	PAIRS TESTED F ₂	PAIRS STERILE F ₂	STERILE ♀	STERILE ♂
55	13	0	55a	3	0	0	0
56	9	0	56a	51	3	2	
57	13	0	57a	16	1	1	0
58	9	0	58a	22	1	1	0
59	11	0	59a	14	0	0	0
60	11	1♂	60a	24	8	8	0
61	9	0	61a	6	3	2	1
62	4	1?	62a	22	1	1	0
			62b	4	0	0	0
63	8	0	63a	4	2?	1?	1?
			63b	4	0	0	0
64	11	0	64a	50	0	0	0
65	4	0	65a	32	3	1	2
			65b	6	0	0	0
			65c	5	2		
66							
67							
68							
69							
70							
71							
72							
73							
74*							
75*							
76*							
77*							
Total	102	2		262	24	17	3

* Sterile

This experiment brings out the following facts. In the sterile female stock (inbred) used as a control 39 pairs out of 110 pairs were sterile. In the Woods Hole stock 4 pairs out of 70 pairs were sterile. In the F₁ generation only 12 pairs in 351 proved sterile. Of those tested 5 were females, 4 were males. In the F₂ generation 1286 pairs were tested, 195 of which were sterile. There were 153 sterile females and 15 sterile males. The sterility of the female reappeared in both the cross and the reciprocal, indicating that both the brothers and sisters of the affected

females were capable of transmitting the defect. The control shows that family No. 151 of the inbred stock was the least affected, and it is to be noted that sterility reappears among the grandchildren of this family in less intensity than among the grandchildren of the other two families. This fact seems to indicate that the intensity to which the sterility reappears in the F_2 generation bears a causal relation to the intensity in which it entered the cross.

Crosses between inbred, truncate and Woods Hole stock

In the sixth generation of the inbred stock, when the sterility of the females appeared in its greatest intensity (50 per cent; table 2), crosses were made with the Woods Hole stock and also with the truncate stock. The truncate stock was also crossed with the Woods Hole stock, and since the truncate stock at this time was a relatively fertile stock, it serves as an excellent control for the female sterility of the inbred stock. This experiment then consisted of three crosses, together with their reciprocals and controls: (1) The inbred stock by the Woods Hole stock; (2) The inbred stock by the truncate stock; (3) The truncate stock by the Woods Hole stock.

The control on the Woods Hole stock used in this experiment shows that one sterile male occurred in 58 pairs (table 12, Lot A).

TABLE 14a

Crosses between the inbred stock and the Woods Hole stock, showing the behavior of sterility in the F_1 and F_2 generations. Woods Hole ♀ × inbred ♂ No. 31

NO. P_1	PAIRS TESTED F_1	PAIRS STERILE F_1	ANCESTRY NO.	PAIRS TESTED F_2	PAIRS STERILE F_2	STERILE ♀	STERILE ♂
36*							
37	22	1	37a	17	0	0	0
			37b	13	0	0	0
			37c	16	0	0	0
38	4	0	38a	10	0	0	0
39	1	0	39a	16	2	1	1
40	0						
Total.....	27	1		72	2	1	1

* Sterile ♂

The truncate stock shows only a slight degree of sterility (table 8, F_{10}). The crosses themselves bear out this statement, for, out of the 24 pairs, all are fertile (table 16). Two died, however, before their fertility could be established and are not recorded in the table. Of the 251 pairs tested in the F_1 generation there are only 2 sterile individuals; a male and a female. The behavior of the sterility is the same in the cross as in the reciprocal. If the sterility behaves like a character which segregates and recombines

TABLE 14b

Woods Hole ♂ × inbred ♀ No. 31; reciprocal 14a

NO. P_1	PAIRS TESTED F_1	PAIRS STERILE F_1	ANCESTRY NO.	PAIRS TESTED F_2	PAIRS STERILE F_2	STERILE ♀	STERILE ♂
41	0	0	0	0	0	0	0
42	7	0	42a	12	7	4	
43	24	0	43a	24	4	2	1
			43b	20	0	0	0
			43c	40	3		1
			43d	33	6	3	
44*							
45*							
Total.....	31	0		129	20	9	2

* Sterile ♀

TABLE 15a

Crosses between the inbred stock and the truncate stock showing the behavior of sterility in the F_1 and F_2 generations. Inbred ♀ No. 31 × truncate ♂ No. 35

NO. P_1	PAIRS TESTED F_1	PAIRS STERILE F_1	ANCESTRY NO.	PAIRS TESTED F_2	PAIRS STERILE F_2	STERILE ♀	STERILE ♂
25	16	0	25a	16	6	3	1
			25b	36	0	0	0
			25c	28	9	4	4
			25d	18	6	4	2
			25e	33	3		
26*							
27*							
28*							
29*							
Total.....	16	0		131	24	11	7

* Sterile ♀

TABLE 15 b

Inbred ♂ No. 31 × truncate ♀ No. 35; reciprocal 15 a

No. P ₁	PAIRS TESTED F ₁	PAIRS STERILE F ₁	ANCESTRY NO.	PAIRS TESTED F ₂	PAIRS STERILE F ₂	STERILE ♀	STERILE ♂
31							
32							
33	8	0	33a	46	4	2	
			33b	29	15	7	1?
			33c	48	9	7	
34	19	0	34a	24	1	1	0
			34b	39	3	2	1
			34c	12	5	2	3
35	11	0	35a	48	6	3	1
			35b	31	3	1.	
			35c	38	22	9	1?
Total.....	38	0		315	68	34	7

TABLE 16 a

Crosses between Woods Hole stock and truncate stock showing behavior of sterility in F₁ and F₂ generations. Woods Hole ♀ × truncate ♂

NO. P ₁	ANCESTRY NO. P ₁	PAIRS TESTED F ₁	PAIRS STERILE F ₁	ANCESTRY NO.	PAIRS TESTED F ₂	PAIRS STERILE F ₂	STERILE ♀	STERILE ♂
18	35	10	0	18a	7	1	1	0
				18b	14	0	0	0
19	35	15	0	19a	22	2	2	0
				19b	12	3		
20	26	9	0	20a	15	8	4	4
				20b	17	2	2	0
21	26	18	0	21a	19	0	0	0
				21b	25	1?		
				21c	8	0	0	0
22	35	11	0	22a	24	2	1?	1
				22b	32	4	4	0
23	35	9	0	23a	7	0	0	0
				23b	5	0	0	0
24	35	22	1 ♀	24a	23	0	0	0
				24b	13	2	1	1
		54*	0					
Total.....		148	1		243	25	15	6

* Pairs chosen at random from the above crosses.

in the germ plasm we should expect it to reappear only slightly intensified in this case among the grandchildren. A study of the 528 pairs tested in the F_2 generation verifies the expectation. This case shows that sterility as it reappears among the grandchildren bears a more or less definite relation to the degree to which it was put into the cross. The behavior of the sterility of the truncates at this time is significant for their earlier history shows that half the pairs were sterile. As stated, it was at first my opinion that sterility was inherent in the strain, and that it bore a causal relation to low productivity. But the above results seem to show that sterility and low production of fertile brothers and sisters are two entirely different things. Such a

TABLE 16 b
Woods Hole ♂ × truncate ♀; reciprocal 16 a

NO. P_1	ANCESTRY NO. ♀ P_1	PAIRS TESTED F_1	PAIRS STERILE F_1	ANCESTRY NO.	PAIRS TESTED F_2	PAIRS STERILE F_2	STERILE ♀	STERILE ♂
1	35	13	0	1a	12	3?	2?	1?
				1b	25	1	0	1
2	35		0		0	0	0	0
3	35	10	0	3a	14	0	0	0
				3b	7	1	1	0
4	35	12	0	4a	16	1?	1?	0
				4b	20	1	1	0
5	35	25	0	5a	20	2	2	0
				5b	19	0	0	0
6	35							
7	35							
8	33	4	1♂	8a	14	1	0	1?
				8b	38	1	1	0
9	33							
10	26	11	0	10a	19	2?		1
				10b	6	2?		1
				10c	17	0	0	0
11	26	7	0	11a	8	1	0	1
				11b	11	0	0	0
12	33	8	0	12a	13	0	0	0
				12b	26	11	0	11
13	34							
14	34	5	0					
17	35	8	0					
Total.....		103	1		285	27	8	17

view paved the way for a study of the low production of the truncates, which is dealt with in the second part of this paper.

It is to be noted in 12 b (table 16 b) of this cross that 11 pairs out of 26 are sterile. This proved to be due to the males. Twenty-eight other males from this family were tested with the hope of establishing a sterile male strain. Seventeen of the 28 were sterile. This gave promise of yielding a sterile male line; but on inbreeding a few pairs the sterility vanished as suddenly as it had appeared. This is the only case in all the experiments in which sterility affected the males to any appreciable degree.

Let us turn now to the evidence which this experiment gives on the transmission of female sterility. A study of the tables where the sterile female strain (inbred stock) was tested against the truncate and Woods Hole stocks shows that the sterility reappeared again after skipping a generation and that it affected primarily the females. The defect reappears in both the cross and the reciprocal. A great deal of weight is to be attached to this evidence since the sterility did not appear to any appreciable degree when the truncate stock was crossed to the Woods Hole stock. Moreover, the sterility as it was affecting the female was now at its maximum. In table 14 a there are three inbred males that did not transmit the defect. An apparent explanation is found on the assumption, which is borne out by all the evidence presented, that the defect behaves like a unit character and accordingly some families are not affected.

Crosses between inbred and truncate stock

Before proceeding to a summary of these sterile cases I wish to give the results of an experiment in which the inbred stock was crossed into the truncate stock. Sterility was present in both stocks at this time. Tables 17 a and 17 b give the result of the sterility as it appeared in the F_1 and F_2 generations.

Table 1, F_1 , gives the control for the inbred stock while table 8, F_{10} , gives the control for the truncate stock. The inbred strain up to the time this cross was made had been relied upon as a fertile strain. That sterility had appeared in this stock and

TABLE 17a

Crosses between the inbred stock and the truncate stock showing the behavior of sterility in the F_1 and F_2 generations. Inbred ♀ × truncate ♂

NO. P_1	NO. PAIRS TESTED F_1	NO. PAIRS STERILE F_1	NO. PAIRS TESTED F_2	NO. PAIRS STERILE F_2	STERILE ♀	STERILE ♂
18	3	0	7	1		
19	3*	0	6	2		
20	6	0	9	3		
21	2	0	0	0		
22*						
23*						
24	4	0	9	3	1	2
25*						
26*						
27*						
28*						
29	8	1	33	0		
30	6	0	14	3		
31	0	0	0	0		
32	8	0	3	1		
33	1	0	0	0		
34	7	0	3	1		
Total.....	48	1	84	14	1	2

* Sterile

TABLE 17b

Inbred ♂ × truncate ♀; reciprocal 17a

NO. P_1	NO. PAIRS TESTED F_1	NO. PAIRS STERILE F_1	NO. PAIRS TESTED F_2	NO. PAIRS STERILE F_2	STERILE ♀	STERILE ♂
1	7	1	4	3		
2*						
3	6	0	8	5		
4	0	0	0	0		
5	6	0	22	4		1
6	0	0	0	0		
7	10	0	4	4	4	
8*						
9	11	0	11	5		
10	13	2	28	3	1	
11*						
12	7	0	17	7	0	2
13	4	0	2	0		
14	6	0	3	1		
Total.....	70	3	99	32	5	3

* Sterile

that it was also present in the truncate stock at this time is shown not only from the controls but also from the crosses themselves. It looks at first sight as if the sterility reappeared in a higher degree in the reciprocal than it did in the cross, since it occurs in 32 cases out of 99 in the reciprocal while in the cross the sterility occurs in 14 pairs of the 84 pairs tested. It is to be noted, however, that the pairs as made up are not equally distributed, for, number 29 of the cross has 33 representatives none of which are sterile. If correction is made for this we have 14 sterile pairs in 51 or 28 per cent which corresponds fairly well with the 33 per cent of sterility as it occurred in the reciprocal cross. The fact that 33 pairs of number 29 showed no sterility is significant as has already been pointed out; for, it shows that some families do not show the defect, while in closely related families the defect may be present to a high degree.

In table 18 I have compiled the results of all the experiments that deal with the transmission of sterility. A census of the whole situation shows that of 417 pairs set aside as controls 113 pairs were sterile. Twenty-seven per cent of the controls were sterile. There were 84 sterile females and 14 sterile males. Of the 149 crosses made between the sterile and fertile strains 35 pairs, or 24 per cent, were sterile. If the sterility behaves anything like a recessive character the expectation is that sterility will not appear in the F_1 generation. A total of 832 pairs tested in the F_1 generation verifies the expectation as only 19, or 2.3 per cent, are sterile. These seem to be chance occurrences as both sexes are equally affected—5 females and 6 males. If one could find an absolutely fertile race against which to test the sterility, it is altogether probable that no sterility at all would appear in the F_1 generation. The sterility as it appeared here in the F_1 generation apparently gives a fair measure of the degree to which the sterility entered into the experiment from the strain against which the sterility of the sterile strain was tested.

In the F_2 generation we find that the sterility as it affects the female reappears. Of the 2644 pairs tested, 407 pairs, or 16 per cent, proved sterile. There were 237 sterile females and 60 sterile males. The rather large number of males given here in-

cludes 15 sterile ones that occurred in one family and was probably due to a chance combination.

A glance at the summary given in table 18 will show that the sterility as it affected the female is practically the same whether inherited through the male or female. I think that the 574 cases of sterility recorded in this summary of 4042 pairs tested gives a faithful picture of the actual facts in the case as seen on a minor scale in each of the experiments and in the history of the inbred stock. The evidence is conclusive that sterility as it affects the female may be present in different intensities in the different families and that female sterility is transmitted by the fertile brothers and sisters of the affected females to the granddaughters but not to the grandsons. This character behaves like a recessive Mendelian character in that it disappears in F_1 and reappears in F_2 .

CROSSES INVOLVING A SEX-LINKED FACTOR

In this experiment, which unfortunately was not carried out on a very extensive scale, the white-eyed stock was used. This stock arose from a mutant of the inbred stock. Females were

TABLE 19

Behavior of sterility in F_2 generation of No. 12 b; control of the supposed sterile male strain

	NUMBER							TOTAL
	1	2	3	4	5	9	11	
Number pairs mated....	2	4	8	12	3	11	3	43
Number pairs sterile....	0	0	1	0	0	2	0	3

paired with the fertile males from No. 12 b, table 15. The reciprocal cross was not made. Family No. 12 b arose as a cross between the Woods Hole and truncate stocks. One-half of the males from this particular combination were sterile. It was my hope that this would develop into a male-producing strain; but the sterility vanished suddenly.

A number of flies from No. 12b were allowed to breed in order to obtain the next generation. From this sixteen pairs were

drawn off and tested in F_1 generation. Much to my surprise, all proved fertile. From 5 of these, seven pairs were made up in the F_2 , as shown in table 19.

As a control of the white-eyed stock eight pairs were tested all of which were fertile.

Fifteen males, brothers of the sterile males whose fertility had been established, were crossed with 15 white-eyed females; all the pairs proved fruitful. Offspring from five of these crosses were paired as shown in table 20. All the males represented in the 62 pairs in the table have white eyes like the mother, while all the females have red eyes like the father, since this is a case of sex-linked inheritance. On inbreeding, four classes of offspring will be produced in the next generation: (1) white-eyed males; (2) red-eyed males; (3) white-eyed females; (4) red-eyed females. The four classes were about equally represented. The virgin females were placed with a number of males; the males with a number of virgin females. The result is given in table 21.

TABLE 20

Sterility as it appeared among the children of the crosses; F_1 generation

	NUMBER					TOTAL
	1	2	3	4	5	
Number pairs tested...	16	21	5	6	13	62
Number pairs sterile...	3	9	0	1	0	13

TABLE 21

Sterility as it appeared in the F_2 generation. Offspring from two of the 21 pairs of No. 2, table 20

Family No. 2 a					Family No. 2 b				
	CLASS					CLASS			
	Red ♂	Red ♀	White ♂	White ♀		Red ♂	Red ♀	White ♂	White ♀
Number tested....	18	19	10	10	Number tested....	14	21	13	20
Number sterile....	3	0	1	0	Number sterile....	2	10+1?	1?	13+2?

The figures given in this experiment are very small and the source of the sterility doubtful since the controls throw no light upon it. It is to be noted, however, that both classes of females are affected in family No. 2 b. In the case of the white-eyed females more than 50 per cent are affected.

To analyze the evidence from the foregoing experiments let it be assumed that a gene responsible for the functioning of the oviduct is carried by the X-chromosome and that in the sterile individuals this gene has changed so that the oviduct fails to function. In analogous cases when the female is affected both X's are affected but she remains normal so long as only one X is affected. When the affected X goes into the males they should not be sterile because the male has no oviduct. But such a male will be able to transmit the defect. To follow the argument, let us make three assumptions.

a. If the defect in this experiment came from the white-eyed grandmother then her germinal make-up would be expressed by the formula wXx , in which the x represents the affected chromosome. Since the red-eyed male did not contain the defect his formula is RXO . Crossing these two individuals gives:

$wX - wX$	
$RX - O$	Gametes
<hr/>	
$wRXx - wRXx - wxO - wXO$ F_1 Red-eyed females and white-eyed males, none of which show sterility.	

If by chance the flies chosen have affected genes, we get:

$wX - RX$	
$wX - O$	Gametes
<hr/>	
$wXx - wRXx - wxO - RXO$ Flies of the F_2 generation; only white-eyed females are sterile	

But this does not agree with the facts of the experiment.

b. If the defect was brought in by the red-eyed grandfather and the female is free from it, then it follows:

$wX - wX$	
$Rx - O$	Gametes
<hr/>	
$wRXx - wXO$ F_1 Red-eyed females, white-eyed males; all fertile.	

On mating the F_1 the following results:

$w X - R x$	Gametes
$w X - O$	
<hr/>	
$w w X X - w R X x - w X O - R x O$	F_2 White-eyed females, red-eyed females, white-eyed males, red-eyed males; no sterile individuals.

This does not accord with the sterility as shown in this experiment.

c. If the defect is brought in the X-chromosome of both grandparents, it follows:

$w x - w X$	Gametes
$R x - O$	
<hr/>	
$w R x x - w R X x - w x O - w X O$	F_1 Red-eyed females, white-eyed males. Half of the females sterile.

On mating the F_1 the following results: *

$w X - R x$	Gametes
$w x - O$	
<hr/>	
$w w X x - w R x x - w X O - R x O$	F_2 White-eyed females and red-eyed females; white-eyed males and red-eyed males; red-eyed females sterile.

This accords with the appearance of the eye colors but not with the appearance of the sterility, since there was practically none in F_1 .

These three assumptions exhaust the possibilities on the hypothesis that the defect is carried by the X-chromosome and yet none of them meets the facts in the case of this experiment. I conclude, therefore, that sterility as it affected the female in this experiment was not transmitted by the X-chromosome. There is a possibility, however, that the factor involved is in the sex chromosome but so far from W that it crosses over freely with it.

DISCUSSION *

The foregoing series of experiments brings out the following facts. (1) Sterility as it appeared in the inbred stock affected primarily the females. The males may also be sterile but it seems probable that the sterility of the male bears no causal relation to the sterility that affects the female. (2) The defect is germinal

in character in that it is strongly transmissible through inheritance, behaving like a character that segregates. The evidence goes to show that the character in all probability is not carried by the X-chromosome. (3) The sterility as it affects the female can be eliminated by selection. Inbreeding, as such, does not appear to be the causative agent in producing this kind of sterility.

A close study of the females revealed the fact that they were not laying eggs. The abdomen swelled until it was very large and in many cases the ovipositor protruded some distance beyond the body. The female could not deposit her eggs. A score or more females were sectioned and these sections showed eggs present in abundance. In fact, a mere examination of the fly after it had been mashed on a glass slide showed in all cases, apparently well-formed eggs in large numbers. I have examined scores in this manner after they had been tested with a number of males and except only one doubtful case I have found that all had eggs. The exact nature of the defect cannot be stated until more attention is given to a study of sections, but the defect is of such a kind that it prevents her from laying her eggs. The possibility that parasites were the source of the trouble suggested itself. Sections show, however, that these flies are remarkably free from parasites in so far as one can make out with ordinary stains, and the behavior of sterility in transmission, moreover, shows the futility of such an hypothesis.

In the case of the fertile female one has merely to bring pressure to bear upon the abdomen to cause her to expel an egg. The sterile females will not respond to such treatment. On the contrary, the wall of the abdomen will burst, and allow the eggs to roll out, while none will pass through the oviduct. Whether or not the eggs are ever fertilized I cannot say. I have frequently seen the sterile female copulate and I have planted scores of eggs (obtained by opening the female) on banana but never has a single larva emerged.¹ All attempts at artificial fertilization proved a failure.

¹ A fertile female placed on a very poor grade of banana may refuse to lay her eggs for some time. She holds them as long as she can but after a few days will lay them.

It is to be recalled that Castle in his cultures found both males and females sterile. Moenkhaus found in his strain, in 64 cases tested, that the males alone were sterile.² Moenkhaus in attempting to harmonize Castle's results with his own points out that the two cases can not with certainty be compared since Castle took no account of the emergence of larvae but merely the production of pupae. In other words, Castle's measure of productiveness was eggs that gave rise to pupae; Moenkhaus's measure, the emergence of larvae. From my studies this difference seems rather apparent than real since in only one or two very questionable cases have I found that larvae after emerging from the egg were unable to develop to the adult stage. I do not wish to be misunderstood on this point. Not every zygotic combination, as is evident from my later studies, results in the production of a well formed fly. In fact, the percentage, as I have been able to show, may be very low. Whether the sperm enters the egg, or having entered the combination dies, is another question. The fact holds in my strains that if the larvae emerge some of them develop to the hatching stage.

I wish to point out that my results and those of Castle are more likely to be harmonized on the assumption that a distinction exists between fertility and sterility. Castle and Moenkhaus seemed to consider that the defect related to sperm and egg structure. I infer from Castle's paper that he supposed that sterile females occurred more frequently in a strain inclined to low productivity; that low fertility and complete sterility as it affects the female are causally related. I interpret him to mean that there is a range in the capacity of the eggs for fertilization by good sperm, extending from zero (or complete sterility) to high fertility. In my strain no such condition is involved; but it is conceivable that the egg-laying power ranges in degree from zero to complete productivity. If so some of the sisters of the sterile females

² He mentions one exception which from the present standpoint seems significant. "In one instance I found among a brood, beside a sterile male, two females that failed to deposit eggs although eggs were evidently present in the oviducts." It seems likely that this is the same defect that I have found so prevalent in my cultures.

should be low producers. This would seem to correspond to Castle's results but in my case we meet with a difficulty at the outset on such an assumption for it appears that the egg-laying power of the affected female is totally abrogated. My evidence will not deny the possibility of transitional stages but it is not clear how an egg-laying mechanism should be able to expel a few eggs and not all.

Critical evidence is hard to obtain on this point because low production may be due to a variety of causes. Attention is called to the truncates, in which there was no rise in productivity as the sterile individuals were practically eliminated.

The history of the inbred stock allows us to form a more reliable opinion, for the total output of offspring at first was much greater. In table 2, Part 2, we get results that look as though the productivity is running down as the sterile females appear. The table, however, does not convey all the facts of the experiment, for in the F_1 generation where 65 offspring is recorded as the average per pair, some of the flies failed to emerge from the pupae. The generations which precede and follow show more nearly the actual facts in the case, namely, a gradual decrease in productivity. That this was a high producing strain in the beginning of the experiment there can be no doubt; for in the second generation the average for sixteen pairs is 365 offspring. The low production in the first generation is probably due to inexperience in handling, as half of the females were dead at the end of two weeks. The production gradually fell despite the fact that facility in handling became more and more perfected. In the F_{14} generation the production had dropped to 159 per pair despite the fact that the sterile females had been practically eliminated. This led to an investigation of the defect. It was found by isolating the eggs of the females of the F_{14} generation, which had been placed with a number of their own males, that only 32 per cent hatched. And yet in the same experiment under identical conditions 58 per cent of the eggs of the F_{14} female hatched when paired with the males from the truncate stock. Moreover, the males of the F_{14} generation were able to fertilize 52 per cent of the eggs of the females of the truncate stock.

These facts make it evident that incompatibility of some kind had arisen between the egg and sperm of the inbred stock. It is not evident that this is in any way related to the appearance of the affected females in this strain.

In regard to the method of transmission of the sterility as it affects the female, Mast, one of Castle's students, came to the conclusion that "An entirely fertile male may transmit partial or complete sterility of the female sex as a racial character to his granddaughters though not apparently to his daughters." Since Castle and Mast's paper deals primarily with the effects of inbreeding, the data upon which these conclusions are founded are very small. As will be seen from my tables, this method of transmission has been verified many times, the exception to be taken here is that in all likelihood the partial fertility bears no relation to the complete sterility. The reciprocal cross that led Mast to the above conclusion

. . . . resulted in one case in the production of females half of which were sterile and the other half were of low productiveness. This case shows that a female of a race inclined to sterility may transmit that character directly to her cross-bred offspring. . . . This difference in heredity through the two sexes would seem to indicate that sterility of the female is dependent upon egg structure, the eggs produced by mothers of a fertile race always yielding fertile daughters. But the eggs of cross-bred females, whose father was of an infertile race, produce some of them fertile, some infertile females.

Such a method of transmission is not borne out in my cases. The females transmit sterility to the granddaughters in the same way as the males and it is certain that the defect skips a generation. Castle and Mast's paper gives us no certain proof as to the defect in the female. It seems to be assumed that she is laying eggs but that these eggs are incapable of fertilization. If this is true her sterility cannot be compared at all to that of the females in my case. In all my experiments, however, I have never found but one or two questionable cases in which the female having laid her eggs proved sterile. In fact, toward the end of the experiments it was easy to tell which sex was at fault by observing whether or not the female was laying her eggs. While this was never made the final test it always held good that when the female

laid eggs which did not develop the male was found to be at fault. Some of the eggs of such a female always hatched on placing her with fertile males. It may or may not be that Castle's case and mine are similar since they behave somewhat differently in heredity. Since Castle's paper deals primarily with another problem, the data upon which the above conclusions in regard to the method of transmission are based are too small to base any definite conclusions upon, since the influence that may have entered into the experiment from the other sex is not with certainty under control. A glance at Mast's last conclusion will show that the male also may have been at fault.

CONCLUSIONS

1. One kind of sterility in *Dròsophila ampelophila* is due to some defect, probably in the oviduct of the female, so that she is unable to deposit her eggs.

2. The defect is transmissible through inheritance by at least some of the brothers and sisters of the affected females when mated to a fertile race, to the granddaughters, but apparently not to the sons or daughters or grandsons. It is therefore recessive and affects females only.

3. The process of inbreeding brothers and sisters cannot be held to be responsible for this condition, but probably serves to bring it out when latent in a strain by making the necessary combinations.

4. The character seems amenable to selection and can be made to affect fully 50 per cent of the females or can be practically eliminated by making the proper selections.

5. It seems very probable that sterility as it affects the male bears no causal relation with sterility as it appears in the female.

6. The defect in the female behaves after the manner of a Mendelian character in that it reappears after skipping a genera-

tion. The normal function is dominant to the negative condition. An unaffected male can transmit something as a dominant character which causes the normal egg deposition of his daughters.

7. The defect is sex-limited in the sense that it affects the female only but it is probably not sex-linked in the sense that it is carried by the X-chromosome. If so, it is such a distance from W that it crosses over more or less freely with it.

I wish to express to Professor Morgan my appreciation of his interest throughout the present study. I am also grateful for the generous gift of the Dyckman Fund which the Department voted me for 1912-1913.

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FERTILITY AND STERILITY IN DROSOPHILA AMPELOPHILA

II. FERTILITY IN DROSOPHILA AND ITS BEHAVIOR IN HEREDITY

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From the Zoölogical Laboratory, Columbia University

NINE FIGURES

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INTRODUCTION

The first study demonstrated that the sterility there dealt with is due to some defect probably in the oviduct of the female that prevents her from laying eggs. Since this defect is transmitted by males and heterozygous females, the defect when once in a strain constantly recurs. It is evident that this kind of sterility in the female is an entirely different thing from infertility in the sense that eggs laid are not fertilized or if fertilized do not develop.

It is evident from Part I that the low fertility of the truncate stock would not be explained by the presence of the sterile individuals for after sterility had been practically eliminated the

truncates produced no more offspring per fertile pair than at the beginning of the experiment when half of the pairs were sterile. Neither can the low productivity of the truncates be explained entirely because of the shortened length of life, although this is one factor in the result.

The truncates differ in many ways from the wild stock:

1. The flies give rise to a small number of offspring per pair. Table 1 gives the number of offspring produced through several generations of inbreeding. Despite all the care in breeding that could be exercised no way was found to increase the output.

2. The truncate flies do not live as long as the wild fly. The length of life is in general about half that of the inbred stock which was used in the control. The two strains were bred under identical conditions (compare tables 1 and 2).

3. Truncates, as the name implies, have the ends of the wing squared instead of rounded. The wings extend only to the tip of the abdomen. The flies will not breed true, for they produce

TABLE 1
History of truncate stock through seventeen generations of inbreeding

	GENERATION											
	F ₁₃	F ₁₄	F ₁₅	F ₁₆	F ₁₇	F ₁₈	F ₁₉	F ₂₀	F ₂₁	F ₂₂	F ₂₃	F ₂₄
No pairs tested.....	31	53	46	19	1	14	21	2	2		8	15
No pairs sterile.....	22?	27	12	6	0	2	3	0	0		1	2?
No pairs from which off- spring were counted. . .	9	26	34	13	9	9	8				7	13
Truncate ♂.....	173	855	684	266	—	79	182				182	218
Truncate ♀.....	212	911	696	292	—	99	202				162	228
Long ♂.....	41	123	136	29	—	10	37				31	66
Long ♀.....	43	117	102	32	—	14	25				24	57
Total ♂.....	214	978	820	295	14	89	219				213	284
Total ♀.....	255	1028	798	324	14	113	227				186	285
Grand total.....	469	2006	1618	619	28	202	446				399	569
Average.....	52	77	47	48	28	22	56				57	51
Average life of ♂ in days.....	—	—	26	27	—	26	26				27	23
Average life of ♀ in days.....	—	—	16	25	—	17	25				19	17

some offspring with wings like those of the wild fly; at least this has held true through many generations of inbreeding truncate brothers and sisters. The long winged flies from truncates throw in turn some truncates.

HISTORY OF THE LOW-PRODUCING TRUNCATE AND THE HIGH-PRODUCING INBRED STOCKS

In order to bring into sharp contrast the difference that exists in the productivity of these two strains I have compiled in tables 1 and 2 the data of these two strains through several generations of inbreeding. The truncates gave on an average about 50 offspring per pair, and the inbred stock about 200 offspring per pair. It has been stated that the inbred flies live longer than the truncates, but this difference, as the tables show, will not account for the marked difference in production.

Table 2 gives the history of the wild inbred stock described in Part I. This was bred parallel to the truncates in table 1 and was subject to exactly the same environmental conditions.

TABLE 2
History of the inbred stock

	GENERATION													
	F ₁	F ₂	F ₃	F ₄	F ₅	F ₆	F ₇	F ₈	F ₉	F ₁₀	F ₁₁	F ₁₂	F ₁₃	F ₁₄
No. pairs tested	16	16	7	12	10	105	34	100	40	11	182	12		16
No. pairs sterile	1?	0	3	3	2	51	15	39	6	0	28	1		1
No. pairs from which offspring were counted.....	13	13	4	9	8	11	11	—	—	—	—	—	—	14
Males produced	975	2374	439	854	708	336	629	—	—	—	—	—	—	1098
Females produced.....	1034	2406	395	859	766	310	677	—	—	—	—	—	—	1142
Total.....	2009	4780	834	1713	1474	646	1306	—	—	—	—	—	—	2240
Average.....	155*	368	209	190	184	65†	119	—	—	—	—	—	—	159
Days ♂ lived...	—	45	42	41	36	45	34	—	—	—	—	—	—	45
Days ♀ lived...	—	37	35	33	37	35	30	—	—	—	—	—	—	26

* The low production in this case was due to inexperience in handling. Half of the females were dead at the end of the second week.

† Some of the flies failed to emerge from the pupae.

CROSSES BETWEEN THE LOW-PRODUCING TRUNCATE STOCK AND THE HIGH-PRODUCING WILD STOCKS

The following experiments were carried out in order to determine whether the egg or sperm was responsible for the low fertility of the truncates.

Females from the low-producing truncate stock were paired to males from the high-producing inbred stock. The reciprocal cross was also made. Since the number of offspring bears a more or less direct relation to the length of life of the pairs, data on the length of life is also given. Tables 3, 4 and 5 give the result of the first experiment.

This experiment shows that the low-producing truncates were producing an abundance of fertile sperm, since each was capable of fertilizing on the average 428 eggs of the female of the high-

TABLE 3a

Showing crosses between the low-producing truncates and the high-producing inbred stock

Truncate ♀ × inbred ♂

NO.	CRES- CENT ♂ ^a	CRES- CENT ♀	LONG ♂	LONG ♀	TOTAL ♂	TOTAL ♀	GRAND TOTAL	LIFE OF ♂ IN DAYS	LIFE OF ♀ IN DAYS
1	5	7	101	119	106	126	232	56	26
2†	—	—	—	—	—	—	—	15	41
3	0	0	53	71	53	71	124	15	31
4	—	—	—	—	—	—	—	?	15
5	10	11	146	162	156	173	329	41	13
6†	—	—	—	—	—	—	—	43	8
	2	5	36	54	38	59	97	41	7
8†	—	—	—	—	—	—	—	63	7
10	10	6	185	187	195	193	388	46	26
11†	—	—	—	—	—	—	—	34	13
12	0	0	134	113	134	113	247	35	35
14	6	4	80	61	86	65	151	16	16
16	6	2	78	103	84	105	189	37	24
17	2	0	11	14	13	14	27	44	5
Total.....	41	35	824	884	865	919	1784	496	267
Average.....	—	—	—	—	—	—	198	38	19

^a 'Crescent' as recorded in table 3 are longwinged flies with a small crescent-shaped piece cut out of the inner margin at the end of the wing. They are a new class that arise on crossing the truncates with wild stock.

† Sterile.

TABLE 3b
Reciprocal: Truncate ♂ × inbred ♀

NO.	CRIS- CENT ♂	CRIS- CENT ♀	LONG ♂	LONG ♀	TOTAL ♂	TOTAL ♀	GRAND TOTAL	LIFE OF ♂ IN DAYS	LIFE OF ♀ IN DAYS
18	5	27	332	316	337	343	680	49	59
19	5	4	130	136	135	140	275	?	53
20	0	2	283	335	283	337	620	66	66
21	0	0	191	173	191	173	364	33	?
22*	—	—	—	—	—	—	—	—	—
23*	—	—	—	—	—	—	—	63	63
24	5	17	185	189	190	206	396	26	24
25*	—	—	—	—	—	—	—	41	11
26*	—	—	—	—	—	—	—	33	41
27*	—	—	—	—	—	—	—	34	21
28*	—	—	—	—	—	—	—	65	16
29	2	9	302	293	304	302	606	33	49
30	0	0	86	118	86	118	204	37	37
31	2	4	263	284	265	288	553	36	55
32	0	0	222	249	222	249	471	36	53
33	1	20	127	126	128	146	274	36	67
34	3	1	133	131	136	132	268	10	44
Total.....	23	84	2254	2350	2277	2434	4711	598	659
Average....	—	—	—	—	—	—	428	40	44

* Sterile.

TABLE 4

Control for the high-producing inbred stock used in Experiment I, table 3; compare with table 2.

NO.	TOTAL NUMBER OFFSPRING	MALES	FEMALES	LIFE OF ♂	LIFE OF ♀
10	45	28	17	26+	26+
11	123	59	64	26±	26+
13*	—	—	—	35	26
14*	—	—	—	44	26
16*	—	—	—	40	15
17	311	159	152	40	40
18	227	106	121	15	55
19	198	100	98	40	40
20	340	163	177	66	57
21	46	21	25	26	26
22	192	108	84	66+	34
23	231	115	116	66+	26
Total.....	1713	859	854	490	397
Average....	190	—	—	41	33

* Sterile.

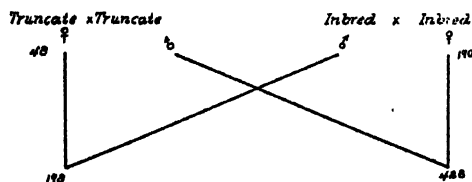


Diagram A Effect on productivity of crossing truncate and inbred stocks; summary from tables 3, 4 and 5.

producing strain, in fact, more than twice as many as when she was fertilized by her own male. The female of the low-producing strain was evidently producing a large number of eggs that were capable of fertilization as shown by the fact that on an average 198 of her eggs were fertilized. And yet the truncates used for control only produced an average of 48 to the fertile pair. The

TABLE 5

Control for the low-producing truncate stock, used in Experiment I, table 3; compare with table 1

NO.	LONG ♂	LONG ♀	TRUNCATE ♂	TRUNCATE ♀	TOTAL ♂	TOTAL ♀	GRAND TOTAL	LIFE OF ♂	LIFE OF ♀
1	2	1	9	13	11	14	25	25 ±	25 ±
2	1	5	6	4	7	9	16	30+	30+
3	6	9	59	83	65	92	157	30+	30+
4	1	0	33	40	34	40	74	23	30 ±
5*	—	—	—	—	—	—	—	34	34
6	0	0	17	19	17	10	36	37	14
7*	—	—	—	—	—	—	—	31	10
8	0	0	7	12	7	12	19	14	14
9	9	13	39	46	48	59	107	30 ±	30 ±
11	0	0	4	3	4	3	7	28 ±	23
12*	—	—	—	—	—	—	—	14	8
13	1	0	1	4	2	4	6	32	8
14	2	2	46	45	48	47	95	42	42
16*	—	—	—	—	—	—	—	32	32
17	0	1	18	10	18	11	29	37	42 ±
18	0	1	2	0	2	1	3	7	16
19*	—	—	—	—	—	—	—	33	19
20	7	0	25	13	32	13	45	27+	27+
21*	—	—	—	—	—	—	—	11	30
Total....	29	32	266	292	295	324	619	517	464
Average...	—	—	—	—	—	—	48	27	25

* Sterile

crosses averaged even better than the high producing strain. Both were mutually benefitted by the cross, but it may seem that the production of the truncate female was limited because she lives on the average about half as long as the inbred female. The truncate male lives on the average about ten days longer,—long enough apparently to fertilize all the eggs of the inbred female. It should be stated that I have verified Castle's observation many times that more than one copulation is necessary to fertilize all of the output of eggs from a female. It seems that the male lives on the average long enough to fertilize this second output of eggs.

It is evident that the short life of the truncate female does not explain entirely her low production, for if the results of this experiment may be relied upon, there was evidently incompatibility of some sort between the egg and sperm in this strain.

The results of this experiment were very surprising in the light of Castle's work. Castle had made crosses between a low-producing strain and a high-producing strain with the result: "that low productiveness (or sterility) of the female may be transmitted directly through the egg from mother to daughter, but only indirectly through the sperm, the character skipping a generation." Castle's conclusion in regard to this case lends support to the assumption which I made in Part I, viz., that he did not distinguish between complete sterility of the female and the low fertility that is here shown.

The problem of the increased fertility of the truncate stock when crossed into a high producing strain was now put to another test. The experiment was carried out in the same way as the previous one except that the Woods Hole stock was used for the high-producing strain, as it had been observed in other experiments to give rise to a rather large number of offspring. Tables 6, 7 and 8 give the result of this experiment.

At first sight this experiment is not as striking an experiment as Experiment I. Neither cross did as well as would be expected from the controls. Nevertheless, the same relation is evident, for while the truncate female produced an average of 56 offspring by her own male, she produced on an average of 118 by the Woods Hole male. It is to be noted, moreover, that her length of life on

the average was only 15 days in the cross while in the control it was 25 days. It seems, not only from this, but from the other evidence, that had she lived on the average ten days longer her output would have been much greater than 118. The truncate male on the other hand was able to fertilize on an average at least 245 eggs of the female of the Woods Hole stock. But this length

TABLE 6a

Crosses between the low-producing truncate stock and the high-producing Woods Hole stock.

Truncate ♀ × Woods Hole ♂

NO.	CROSS- CENT ♂	CROSS- CENT ♀	LONG ♂	LONG ♀	TOTAL ♂	TOTAL ♀	GRAND TOTAL	LIFE OF ♂	LIFE OF ♀
1	11	13	152	169	163	182	345	25	24
2	8	3	28	64	36	67	103	19	13
3	1	0	49	61	50	61	111	15	13
4	2	0	111	108	113	108	221	23	14
5	3	1	20	31	23	32	55	?	4
6	2	0	15	25	17	25	42	29	14
7	1	0	16	19	17	19	36	34	13
8	1	0	28	36	29	36	65	15	28
9	2	0	42	48	44	48	92	23	17
10	3	2	43	62	46	64	110	38	11
Total.....	34	19	504	623	538	642	1180	221	151
Average..	—	—	—	—	—	—	118	25	15

TABLE 6b

Reciprocal: Truncate ♂ × Woods Hole ♀

NO.	CROSS- CENT ♂	CROSS- CENT ♀	LONG ♂	LONG ♀	TOTAL ♂	TOTAL ♀	GRAND TOTAL	LIFE OF ♂	LIFE OF ♀
11	11	13	117	115	128	128	256	17	29
12	3	8	150	159	153	167	320	17	38
13	0	1	67	124	67	125	192	17	23
14	1	0	138	157	139	157	296	21	38
15	5	9	124	155	129	164	293	17	25
16	1	0	56	71	57	71	128	21	19
17	9	18	112	90	121	108	229	18	20
Total.....	30	49	764	871	794	920	1714	128	192
Average..	—	—	—	—	—	—	245	18	28

of life is much shorter than in the controls. In other words, despite all of the unfavorable influences that entered into the crosses in comparison to the controls the point at issue is plainly evident that the female is producing a large percentage of eggs that are capable of fertilization, the male an abundance of good sperm, and yet when the two meet each other there is incompatibility of some sort between egg and sperm, as inferred by the number of offspring produced. It is clearly evident that the length of life is a factor, but by no means the only factor, involved in the low

TABLE 7

Control of Woods Hole stock. Brothers and sisters of the high-producing strain used in the cross in table 6

NO.	TOTAL NUMBER OFFSPRING	MALES	FEMALES	LIFE OF ♂	LIFE OF ♀
1	244	111	133	30	30
2	527	263	264	40	36
3	351	185	166	26	57
4	347	155	192	21	42
5	249	116	133	22	23
6	287	149	138	30	33
7	290	139	151	34	60
8	275	126	149	44	30
Total	2570	1244	1326	247	311
Average	321	155	166	31	39

TABLE 8

Control of truncate stock. Brothers and sisters of the low-producing strain used in the cross in table 6.

NO.	LONG ♂	LONG ♀	TRUNCATE ♂	TRUNCATE ♀	TOTAL ♂	TOTAL ♀	GRAND TOTAL	LIFE OF ♂	LIFE OF ♀
1	1	0	1	0	0	0	2	25	25
2	4	6	36	51	40	57	97	32	32
3	4	3	15	17	19	20	39	21	24
4	1	1	17	19	18	20	38	26	23
5	3	4	15	17	18	21	39	26	17
6	14	7	39	43	53	50	103	31	25
7	8	0	19	21	27	21	48	17	21
8	2	4	40	34	42	38	80	30	30
Total	37	25	182	202	219	227	446	208	197
Average	—	—	—	—	27	28	56	26	25

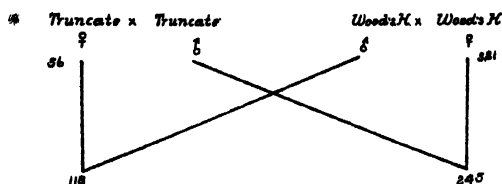


Diagram B Effect on productivity of crossing truncate and Woods Hole stocks; summary from tables 6, 7 and 8.

production of the truncates. To judge from the history of these strains and from Experiments I and II it would seem as though only one egg in four or five of the truncates gave rise to a mature fly when fertilized by its own sperm. I shall not analyze the data further here as I shall later bring forward better evidence that bears on this relation.

FERTILITY OF THE F_1 AND F_2 GENERATIONS FROM THE CROSSES BETWEEN THE LOW-PRODUCING TRUNCATES AND THE HIGH-PRODUCING WILD STOCKS AS DETERMINED BY BREEDING THE ANIMALS TOGETHER IN PAIRS

We may next consider the evidence that bears on the behavior of the hybrids in the F_1 and F_2 generations. In order to determine the productivity of the hybrids from the cross between the truncate female and the inbred male, 65 pairs of the F_1 brothers and sisters were made up from the crosses as given in table 3a. From the reciprocal cross 47 pairs of the F_1 brothers and sisters were paired. The different families were about equally represented. Both F_1 hybrids proved to be most virile animals and excellent producers. In the thirty days from December 22, 1911, when they were first paired as virgin flies, to January 22, 1912, when the experiment was discontinued due to an accident the sixty-five pairs produced a total of 16096, an average of 248 offspring to the pair, and the 47 pairs of reciprocals produced a total of 11800, an average of 251 offspring to the pair. The different classes that appeared are given in table 9.

The experiment shows that the hybrids are excellent producers and that it makes no difference whether the father or the mother was of the low-producing truncate stock. Moreover, it seems as

TABLE 9

Showing the number of offspring produced by 112 pairs of the hybrids in 30 days

	TRUN- CATE ♂	TRUN- CATE ♀	CRES- CENT ♀	CRES- CENT ♂	LONG ♂	LONG ♀	TOTAL ♂	TOTAL ♀	GRAND TOTAL	AVER- AGE
F ₁ (T♀ × I♂)...	516	778	431	633	6881	6857	7828	8268	16096	248
F ₁ (T♂ × I♀)...	273	332	315	436	4986	5458	5574	6226	11800	251

though the hybrids are long lived as shown by the fact that in this experiment at the end of thirty days 81 per cent of the flies were still living. Twenty-four males and 31 females had died. The death rate was not selective but was about equal in both the cross and its reciprocal.

Table 10 gives evidence on the same question—the output of the hybrids as tested in pairs. In this experiment the hybrids

TABLE 10

Showing production of the hybrids of the crosses between the truncate stock and the Woods Hole stock

TABLE 10 a

F₁ generation from truncate ♀ ×
Woods Hole ♂

NO.	TOTAL ♂	TOTAL ♀	GRAND TOTAL	LIFE OF ♂	LIFE OF ♀
1	64	83	147	27	20
2	98	130	228	20	24+
4	96	119	215	27	33
5	103	95	198	54	27
8	143	169	312	24	35±
9	90	117	207	20	24
10	96	110	206	27	33
11	100	140	240	45	24
13	53	67	120	20	15
14	167	138	305	40	27
16	101	139	240	40	33
17	171	186	357	54	54
18	142	150	292	63	20
19	87	100	187	33	49
20	126	146	272	27	33
22	238	244	482	39	49
24	178	215	393	33	40
Total	2053	2348	4401	593	540
Av.			259	35	32

TABLE 10 b

Reciprocal cross: F₁ generation from
truncate ♂ × Woods Hole ♀

NO.	TOTAL ♂	TOTAL ♀	GRAND TOTAL	LIFE OF ♂	LIFE OF ♀
31	55	67	122	45	15
33	92	124	216	57	24
34	92	105	197	40	24?
35	70	94	164	16+	24
37	117	143	260	33	27
38	192	198	390	33	45
40	147	228	375	24	33+
41	99	120	228	37	15
42	54	72	126	24	27
43	104	113	217	12	24
44	106	127	233	40	20?
46	66	79	145	33+	27
47	17	23	40	20	12
49	219	269	488	24	63
50	112	126	238	45	—
Total	1542	1907	3449	483	380
Av.			230	32	27

between the truncates and the Woods Hole stock were used. The hybrids came from the cross given in table 6.

In the experiments given in table 11 the hybrids came from crosses between inbred and truncate stock, as recorded in tables 15a, b, Part I. At this time the inbred stock was in the F_1 generation and was a high-producing strain. The truncate stock was in the F_{48} generation.

The results of these three experiments make it certain that the hybrid offspring from the low-producing truncates and high producing wild stocks are high producers. In fact, the hybrids produce equally as well, if not better, than the high producing parental stock. Moreover, it makes little if any difference whether the father or mother is from the low-producing truncate stock. The following experiments give the evidence that bears on the question as to whether the low rate of fertility of one of the parents will reappear in the F_2 generation.

Tables 12 and 13 give the results of testing in pairs the offspring of the hybrids, derived from the crosses between the low-producing truncates and the high-producing wild stocks. The pairs as

TABLE 11

Hybrids of the crosses between the truncate stock and the inbred stock; the F_1 generation

TABLE 11a

The F_1 generation from truncate
♀ × inbred ♂

NO.	MALES	FE- MALES	TOTAL	LIFE OF ♂	LIFE OF ♀
7	204	205	589	42	39
8	180	224	404	56	41
16	197	228	425	23	55
22	164	176	340	40	45
32	235	215	450	39	33
36	50	61	111	25	61
10	244	246	490	23	73
11	259	242	501	45	62
30	166	208	374	58	44
Total	1789	1895	3684	351	453
Av.			400	39	50

TABLE 11b

Reciprocal: The F_1 generation from
truncate ♂ × inbred ♀

NO.	MALES	FE- MALES	TOTAL	LIFE OF ♂	LIFE OF ♀
1	184	256	440	58	48
6	174	213	387	60	40
9*	91	119	210	16	16
12	120	170	290	80	40
19	121	137	258	73	23
Total	690	895	1585	287	167
Av.			317	57	33

* Died at end of sixteen days because of failure to feed on transfer to new bottle.

given in table 12 came from the hybrids recorded in table 9. The pairs given in table 13a, b came from the hybrids recorded in table 11.

Tables 12 and 13 appear to show that low production reappears after skipping a generation and that this low production is transmitted through both the egg and sperm to the grandchildren. I shall not attempt to analyze the data further as I have other and more exact evidence that bears on the transmission of the low fertility of the truncates.

CROSSES BETWEEN THE LOW-PRODUCING TRUNCATES AND THE
HIGH-PRODUCING WILD STOCKS IN WHICH AN EXACT
MEASURE OF FERTILITY IS EMPLOYED

The term 'fertility' is used in so many different ways by different writers that I wish to make clear at the outset the sense in which the term is used throughout the ensuing papers. By 'fertility,' I mean the percentage of eggs that complete development and give rise to mature flies. For example, if 100 eggs are isolated from a stock and later 50 flies come from these eggs then I speak of the fertility of that stock as 50 per cent. It is evident that fertility in this sense can only be determined by isolating the eggs, and that the number of offspring produced by a pair does not give a measure of the fertility of that pair but only a measure of the 'productivity' of that pair.

The marked difference in the length of life between the truncate stock and the wild strains together with the increased length of life of the hybrid makes it very difficult to draw safe conclusions in regard to the rôle played by the egg and sperm in fertilization in so far as establishing any definite ratios are concerned. In other words the production of offspring cannot be taken as an absolute measure of the fertilizing power of the egg and sperm for as we shall see later in the case of the hybrids, although their production of offspring is very high, their fertility (combination of egg and sperm) is relatively low.

Accordingly, I wish to return to the question of the low fertility of the truncates and consider the behavior of the low fertility in this race on crossing into other races. In order to insure an

TABLE 12

Showing the result of mating in pairs the F_2 generation of the crosses between the short-lived, low-producing truncates and the relatively long-lived high-producing inbred stock

TABLE 12 a

Grandchildren of the cross between the truncate ♀ and inbred ♂

FAMILY NUMBER OF GRANDPARENTS	FAMILY NO. P ₁	NUMBER	NO. OFFSPRING PRODUCED	LIFE OF ♂	LIFE OF ♀
5	34	113	0	24	7
	33	137	5	15	?
	33	147	49	17	36
	34	157	331	38+	38+
	34	161	319	24	29
	33	167	159	14	35
	33	172	106	47	?
7	33	173	330	32	46
	91	118	0	—	—
	91	122	0	—	—
	91	125	0	—	—
12	91	155	0	—	—
	107	114	111	24	21
	107	116	42	42	14
	106	121	50	?	14
	106	123	251	31	31
	50	129	25	12	?
	106	130	29	32	7
	50	134	280	20	39
	107	135	62	9	23
	50	138	164	30	38
	50	146	208	25+	25
	106	150	224	23	30
	106	154	75	20	15
	106	175	178	29	35
	107	182	0	—	—
	107	184	44	21	12
14	117	112	196	26	48
	117	115	0	—	—
	23	139	67	39	?
	26	143	171	36	46
	26	145	0	—	—
	117	158	0	—	—
	117	159	21	55	?
	117	162	8	16	12
	26	180	204	59	46
	26	184	44	21	12
16	80	126	190	35+	35+
	80	151	288	37	32
14	26	183	15	51	?
Av.			140	30—	30—

TABLE 12 b

Reciprocal cross: Grandchildren of the cross between the truncate ♂ and the inbred ♀

FAMILY NUMBER OF GRANDPARENTS	FAMILY NO. P ₁	NUMBER	NO. OFFSPRING PRODUCED	LIFE OF ♂	LIFE OF ♀
20	111	119	74	14+	14+
	111	120	0	—	—
	111	140	256	38	46+
	111	149	0	—	—
	111	153	150	41	44
	111	163	152	5	33
	111	165	0	—	—
	111	178	215	35+	35+
	111	181	135	16	16
	102	111	89	40	18
29	95	117	153	24+	24+
	102	124	192	14	24
	100	127	136	18+	34
	102	128	98	7	7
	95	131	200	34	34
	95	132	200	26	26
	102	133	107	20	23
	100	136	6	38	6
	102	141	129	27	23
	102	142	8	41	6
	102	144	42	39	15
	95	152	46	6	39
	81	156	191	33	?
	102	160	298	38	?
	100	164	432	32	32
30	102	166	379	24	35
	102	168	61	22	22
	102	169	189	29	24
	102	170	288	?	32
	95	171	226	38	26
	102	174	111	24	26
	95	176	205	54	26
	102	177	43	19	22
	102	179	230	38	24
	102	185	425	32	37
Av.	113	148	0	—	—
			171	30—	26—

TABLE 13 a

Table showing result testing in pairs the grandchildren of the crosses between the short-lived low-producing truncates and the relatively long-lived, high-producing inbred stock. T = truncate; C = crescent

Grandchildren of the cross between the truncate ♀ and inbred ♂

FAMILY NO. OF GRAND- PARENTS	FAMILY NO. P ₁	NO.	TOTAL ♂	TOTAL ♀	GRAND TOTAL	LIFE OF ♂	LIFE OF ♀
33	7	415	29	41	70	40	16
		416	18	33	51	45	16
		417 C	23	6	29	52	19
		418	0	0	0	32	6
	8	386	34	37	71	27	27
		387	119	182	301	49	33
		388	21	41	62	15	15
	16	389 T	0	0	0	16	30
		390	0	0	0	46	44
		391 C ♂	67	72	139	27	27
		392 C ♂	73	113	186	39	26
		393	43	37	80	16	16
		394	92	102	194	60	17
		395	77	121	198	61	25
		436 T	29	25	54	15	15
		437	0	0	0	54	15
		438 T	18	20	38	54	11
34	22	432	77	79	156	52	16
		433 C	145	131	276	52	29
	32	397	103	143	246	30	30
		398	71	81	152	53	33
		399	68	72	140	39	20
	36	407	60	74	134	63	27
		408	98	102	200	16	16
		414	20	21	41	16	21
	10	400	151	134	285	34	56
		401	83	69	152	45	31
35	10	402 T ♂	88	127	215	9	37
		403 T	78	73	151	31	27
	11	427	51	63	114	16	20
		428	74	100	174	22	32
		429	71	67	138	?	42
	30	430	62	47	109	50	18
		410	2	2	4	37	56
		434	14	14	28	16	6
		435	0	0	0	50	6
Total.....	—	—	1959	2229	4188	1279	884
Average...	—	—	—	—	135	36	25

exact measure of the fertilizing power of the egg and sperm the experiments were carried out in the following manner.

A female was placed in a bottle with four or five males to insure an abundance of sperm. A small piece of banana was introduced on a piece of paper and on this food the female laid her eggs. Each day the food was removed and the eggs picked off individually with the point of a needle. Each batch of eggs was planted on fresh food and the number of eggs recorded. After a few hours new food was given to the parents. This is a most laborious process so that one cannot carry out as many crosses at a time as might be desired, but this does not invalidate the test as such.

Since the Woods Hole stock was the most fertile one at hand, six females were tested with their brothers as a control. Attention is called to the fact that these females were about ten days old when the experiment began. Eleven truncate females were tested against the Woods Hole male. Fourteen truncate females were tested against their truncate brothers. These counts have led to the results given in detail in table 14, a, b and c.

TABLE 13 b

Reciprocal cross: Grandchildren of the cross between the truncate σ^7 and the inbred φ .

FAMILY NO. OF GRAND-PARENTS	FAMILY NO. P ₁	NO.	TOTAL σ^7	TOTAL φ	GRAND TOTAL	LIFE OF σ^7	LIFE OF φ
25	1	420 T	74	71	145	32	45
		421	40	59	99	19	29
		422	0	1	1	20	20
		423	0	0	0	52	26
	6	424	99	104	203	20	42
		425	51	39	90	52	16
		426	92	92	184	22	45
	12	411	7	0	7	55	11
		412	64	68	132	34	29
		413	17	20	37	50	22
		419	0	0	0	11	52
		404	22	28	60	15	15
		405	82	90	172	62	72
		431	21	36	57	29	52
Total....	—	—	569	518	1087	472	436
Average..	—	—	—	—	91	34	31

TABLE 14

Egg count showing fertility of the truncates stock bred to the Woods Hole stock

TABLE 14 a

Woods Hole ♀ ×		Woods Hole ♂	
NO.	NO. DAYS COUNTED	TOTAL NO. EGGS ISOLATED	NO. NOT CONSIDERED
1	27	227	0
2	28	302	0
3	5	40	0
4	25	424	0
5	3	20	0
6	24	389	0
Total	—	1402	0

TABLE 14 b

Truncate ♀ × truncate ♂

NO.	LIFE OF ♀	TOTAL NO. EGGS LAID	NO. NOT CONSIDERED	NO. CONSIDERED	NO. FLIES EMERGED
18	30	271	120	151	42
19*	23	268	177	91	19
20	28	220	148	72	6
21	5	2	2	0	0
22	36	239	0	239	56
23	57	560	111	449	76
24	42	435	32	403	89
25	32	304	23	281	87
26*	38	360	0	360	86
27	20	295	0	295	69
28	34	282	0	282	114
29	6	13	13	0	0
30	5	0	0	0	0
31†	42	394	0	394	150
Total	—	3249	626	2623	644

TABLE 14 c

Truncate ♀ × Woods Hole ♂

NO.	LIFE OF ♀	TOTAL NO. EGGS LAID	NO. NOT CONSIDERED	NO. CONSIDERED	DO FLIES EMERGED
7	5	17	0	17	8
8	8	93	0	93	58
9	9	31	24	7	6
10	10	20	20	0	0
11	3	2	2	0	0
12	28	226	0	226	148
13	14	179	0	179	68
14	6	1	0	1	1
15	9	21	0	21	8
16*	6	6	6	0	0
17	7	18	3	15	10
Total	—	614	55	559	307

* Escaped.

† This was a long winged female and is not considered in the totals. Note that her fertility was somewhat higher than the average. This will be discussed later.

In this experiment the entire output of eggs of the truncates was collected. The count from the Woods Hole stock does not represent the entire output since these flies were about ten days old when the experiment began. It is true that it is practically impossible to get the entire output of eggs by relying upon an ordinary dissecting microscope without spending an undue amount of time, but as the same method was applied throughout this source of error as it enters into a comparison of the output of eggs of the different races is about constant. I feel quite sure that the counts, as here shown, represent over 80 per cent of all the eggs laid. That many were missed is certain as the food from which the eggs were picked was often kept and later larvae were seen to emerge. In regard to the conditions of the experiment as a test of viability, no objection on the grounds of personal selection can be made, as it is impossible to tell beforehand which eggs are going to hatch. Care must be taken not to allow the eggs to stand too long before picking them as the larva will emerge from the fertile ones while the sterile eggs are left behind to be picked up and considered in the counts.

The question may arise in the mind of the reader as to why 626 eggs are not considered in table 14 b in determining the percentage of eggs which hatched. The explanation is given here since the question recurs in some of the other tables and in succeeding experiments. It is evident from the method used in making the test that a picture is given of the productivity of any single female day by day. The 120 recorded in column "No. not considered"—No. 18 (table 14 b) represents the total number of eggs laid by the female through a number of consecutive days in which none of her eggs hatched. The eggs that failed to hatch were in practically all cases those isolated during the first few days of the experiment, although in a few cases they came in the middle of the count, more frequently however toward the close of the count. This seems a fair way to treat the facts as this may be due to other causes operating other than eggs meeting sperm.

In this case the benefit of the doubt is given to the truncates for if these are considered 749 in 3643, or 21 per cent, hatched. In reality the percentage is not changed much and had this been

known in the beginning much labor could have been saved by picking the eggs from mass cultures, although this would give little insight into the total output of eggs from a single female. The former plan allows us to get much closer to the problem in hand. The results of Experiment IV are given in diagram C.

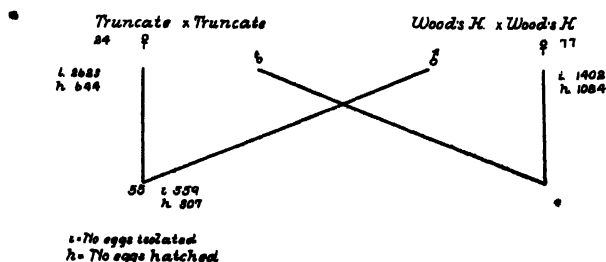


Diagram C Effect on fertility of crossing truncate and Woods Hole stocks; from table 15.

This experiment served in a preliminary way to show that the egg-picking process could be relied upon in general as it gives results in harmony with the earlier breeding experiments. The evidence seems to show conclusively that only one egg in four or five of the truncate female, when fertilized by its own male, will complete development. Moreover, it is shown that the Woods Hole male can fertilize more than twice as many of the truncate eggs than can her own male although it has been shown in earlier experiments that he produces an abundance of fertile sperm. It is interesting to note that only 77 per cent of the eggs of the Woods Hole stock hatched. It was the expectation that this stock would show a fertility of something like 100 per cent. At first I was inclined to believe that this discrepancy was due to some defect in the egg picking process itself, but the results of later experiments together with the behavior of this stock does not bear out any such conclusion. Since these experiments were completed I have obtained a fertility of 96 per cent with two new wild stocks.

It seemed to me that the explanation for the low production of the truncates was not far to seek. That the length of life is a factor is not to be doubted, but more than that it is evident from

the experiments that only relatively few eggs were giving rise to mature flies. I wished to test the conclusion further and also determine whether or not some factor for egg production was operating, as well as to make the cross between the truncate male and the Woods Hole female which combination was not made in the previous experiment.

Experiment V. This experiment was carried out in the same manner as the former one. The flies were mated as soon as they emerged and the output of eggs recorded from each female each day during her life. The facts in detail are recorded in table 15.

The results of this experiment are expressed in diagram D.

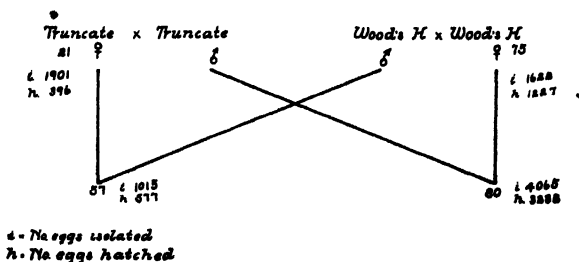


Diagram D Effect on fertility of crossing truncate and Woods Hole stocks; from table 16.

This evidence taken with all the other evidence presented shows conclusively that there is incompatibility between the egg and sperm of the truncates since only one egg in every four or five gives rise to adult flies. It is surprising to find that the truncate male should have actually fertilized more eggs of the female of the Woods Hole stock than a male from its own stock could fertilize, and yet a male from the Woods Hole stock can fertilize almost as many eggs of the truncate female as he can fertilize with a female of his own race. If the results given in diagram D are compared with those obtained in Experiment I as expressed in diagram A; it will be found that the results are in general agreement.

Experiment VI. Reference to table 2, where the history of the inbred stock is given, will show that the fertility has steadily declined on inbreeding, despite the fact that sterile females had increased to 50 per cent and again eliminated. It occurred to me that something like incompatibility between egg and sperm might be operating in this stock

TABLE 15

Egg count showing the fertility of the truncate stock together with the fertility on crossing into the Woods Hole stock

TABLE 15 a

Woods Hole ♀ × Woods Hole ♂

NO.	LIFE OF ♀	TOTAL NO. EGGS LAID	NO. NOT CONSIDERED	NO. CONSIDERED	NO. FLIES EMERGED
24	52	463	0	463	356
25*	51	650	194	456	138
26	33	316	0	316	148
27	52	529	0	529	433
28	25	314	0	314	290
Total	—	2272	194	2078	1365

* Laid very small eggs; some looked as if they might have been parasitized.

TABLE 15 b

Truncate ♀ × Truncate ♂

NO.	LIFE OF ♀	TOTAL NO. EGGS LAID	NO. NOT CONSIDERED	NO. CONSIDERED	NO. FLIES EMERGED
1	32	359	19	340	90
2	12	2	2	0	0
3	23	251	220	31	7
4	24	47	14	33	10
5	4	21	21	0	0
6	35	374	0	374	64
7	7	23	23	0	0
8	6	74	0	74	21
9	30	567	27	540	148
10	49	282	96	186	25
11	32	320	0	320	30
12	6	22	22	0	0
13	19	45	42	3	1
Total	—	2387	486	1901	396

TABLE 15 c

Truncate ♀ × Woods Hole ♂

NO.	LIFE OF ♀	TOTAL NO. EGGS LAID	NO. NOT CONSIDERED	NO. CONSIDERED	NO. FLIES EMERGED
14	14	33	18	15	4
15*	18	0	0	0	0
16	4	0	0	0	0
17	19	265	0	265	214
18†	6	84	0	84	64
19	12	45	39	6	3
20	22	259	6	253	132
21	30	235	53	182	62
22	16	105	0	105	42
23	18	133	28	105	56
Total	—	1159	144	1015	577

* Escaped.

† Copulated.

TABLE 15 d

Woods Hole ♀ × Truncate ♂

NO.	LIFE OF ♀	TOTAL NO. EGGS LAID	NO. NOT CONSIDERED	NO. CONSIDERED	NO. FLIES EMERGED
29	72	730	0	730	617
30	22	323	0	323	276
31*	31	631	0	631	527
32	78	536	17	519	395
33	79	552	18	534	389
34	75	798	12	786	588
35	67	557	15	542	446
Total	—	4127	62	4065	3238

* Escaped.

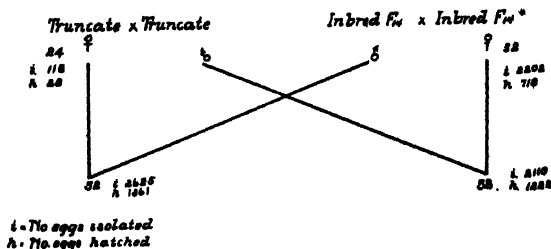


Diagram E Showing the effect on fertility of crossing the inbred and truncate stocks.

and if so this could be put to a test and at the same time more light might be thrown upon the behavior of the truncate stock. Accordingly the inbred stock which was now in the F_{14} generation was crossed into the truncate stock. Table 16 gives the details of the experiment. Table 17 is the control on the inbred stock that was bred in pairs at the same time.

The results of Experiment VI are expressed in diagram E.

BACK CROSSES BETWEEN THE HYBRIDS AND THE RECESSIVE LOW-PRODUCING TRUNCATES

We may next consider the evidence that bears on the fertility of the hybrids when paired with each other and the fertility of the hybrids when back crossed into the recessive low-producing truncates. This experiment was originally undertaken in order to determine whether or not there were sex-linked factors for fertility. The results in this direction have been largely negative but nevertheless the experiments give us the key by which the low productivity of the truncates may be explained on a very simple assumption.

It was my expectation to find that the eggs of the hybrids when isolated would show a fertility of 100 per cent, since I had already found that their production is far in excess of their high-producing parent. Three experiments have been required in order to convince myself that the fertility of the hybrids is in reality low and that the percentage of fertility in the back crosses is an actual measure of the fertilizing power of the egg and sperm.

There are two classes of hybrids, as follows: Offspring from the truncate ♀ by Woods Hole ♂ will be referred to as A ♀ and A ♂.

TABLE 16

Egg count showing the fertility of the inbred stock together with the effect on fertility of crossing into the truncate stock

TABLE 16a

Inbred ♀ × inbred ♂

NO.	LIFE OF ♀	TOTAL NO. EGGS LAID	NO. NOT CONSIDERED	NO. CONSIDERED	NO. FLIES EMERGED
1	28	340	0	340	89
2	21	175	21	154	60
3*	15	325	0	325	68
4	36	400	30	370	116
5	35	403	0	403	174
6†	35	453	20	433	140
7	19	137	3	134	67
8	23	180	137	43	4
Total	—	2413	211	2202	718

* Lived much longer but ceased to lay eggs after 15 days; preserved for study.

† Discontinued.

TABLE 16c

Inbred ♀ × truncate ♂

NO.	NO. DAYS COUNTED	TOTAL NO. EGGS LAID	NO. NOT CONSIDERED	NO. CONSIDERED	NO. FLIES EMERGED
25*	28	291	55	236	111
26*	28	166	12	154	29
27*	26	303	30	273	154
28	27	278	0	278	193
29*	32	107	47	60	18
30*	22	375	0	375	263
31*	22	407	0	407	225
32*	22	327	0	327	229
Total	—	2254	144	2110	1222

* Discontinued.

TABLE 16b

Truncate ♀ × Inbred ♂

NO.	LIFE OF ♀	TOTAL NO. EGGS LAID	NO. NOT CONSIDERED	NO. CONSIDERED	NO. FLIES EMERGED
9	4	0	0	0	0
10	14	18	14	4	1
11	20	221	33	188	88
12	17	112	18	94	45
13	4	0	0	0	0
14	6	0	0	0	0
15	20	101	3	98	38
16	10	130	15	115	38
17	15	122	34	88	46
18	11	96	9	87	39
19	3	0	0	0	0
20	5	45	5	40	32
21*	17	0	0	0	0
22	27	268	0	268	164
23	9	26	12	14	4
24	20	126	14	112	29
33	17	128	0	128	83
34	13	139	5	134	75
37	11	106	0	106	43
38	19	253	0	253	159
39	5	0	0	0	0
40	10	0	0	0	0
41**	17	256	0	256	137
42**	17	251	50	201	123
43	15	126	11	115	41
44†	9	0	0	0	0
45**	17	184	19	165	86
46†	9	0	0	0	0
47	17	72	10	62	39
48††	12	0	0	0	0
49	15	97	0	97	51
Total	—	2877	252	2625	1361

* Escaped.

** Discontinued.

† Killed and preserved for study.

†† Laid a few cheesy looking eggs probably due to parasites.

TABLE 16d
Truncate ♀ × truncate ♂

NO.	LIFE OF ♀	TOTAL NO. EGGS LAID	NO. NOT CONSIDERED	NO. CONSIDERED	NO. FLIES EMERGED
35	26	179	70	109	26
36	8	9	0	9	2
Total	—	188	70	118	28

Offspring from the reciprocal cross will be referred to as B ♀ and B ♂. In these experiments A ♀ is tested with A ♂, B ♀ with B ♂, A ♀ with the truncate ♂, B ♀ with the truncate ♂, the truncate ♀ with A ♂ and the truncate ♀ with B ♂. In order to get an exact measure of the fertility, eggs were isolated day by day from each of the combinations made. The number of eggs isolated each day, and the number of flies that emerged is given in table 18.

TABLE 17

F₁ generation of the inbred stock, showing the result of testing the flies in pairs; compare with table 16a

NO.	NO. OF ♂	NO. OF ♀	TOTAL	LIFE OF ♂	LIFE OF ♀
1	59	67	126	43	26
2	55	39	94	31	31
3	113	133	246	45	37
4	33	31	64	41	10
5	0	0	0	91	13
6	89	90	179	77	21
7	129	139	268	37	37
8	70	90	160	81	17
9	129	133	262	41	35
10	28	31	59	30	24
11	104	111	215	30	28
12	46	46	92	28	24
13	128	96	224	39	28
14	71	59	130	37	25
15	44	77	121	28	28
Total.....	1098	1142	2240	679	384
Average.....	—	—	159	45	26

TABLE 1

gives the number of eggs isolated and the number of flies that hatched by days, from the back crosses between the hybrid (offspring from the high-producing Woods Hole stock and the low-producing truncate stock) and the recessive low-producing truncate stock

COMBINATION																		
NUMBER	A ♀ × T ♂			B ♀ × T ♂			T ♀ × A ♂			T ♀ × B ♂			B ♀ × B ♂			A ♀ × A ♂		
	1-9			10-16			19-31			35-49 17 and 18			50-55			56-61		
	Eggs	Adults		Eggs	Adults		Eggs	Adults		Eggs	Adults		Eggs	Adults		Eggs	Adults	
December 1, '22	176	82	0	148	64	9	207	74	7	200	54	17	—	—	—	—	—	—
2	268	99	0	131	40	17	297	71	12	241	33	41	130	49	0	121	55	0
3	211	110	0	153	67	0	210	74	11	168	35	27	105	57	0	98	34	0
4	141	58	0	95	29	5	132	44	14	129	49	17	91	49	0	100	43	0
5	207	71	0	105	45	46	221	46	6	132	31	54	92	37	27	123	47	0
6	241	88	0	114	30	89	223	58	44	143	41	0	184	77	0	168	70	0
7	136	49	0	122	41	34	121	20	64	100	27	44	121	29	0	149	71	0
8	149	54	0	125	30	47	145	28	33	99	20	14	144	46	0	151	36	0
9	152	48	0	145	32	0	56	12	67	92	24	7	122	35	0	135	39	0
10	158	39	0	148	24	40	58	18	62	66	19	40	126	45	0	131	15	0
11	148	61	0	176	67	0	162	26	—	84	24	—	108	33	0	147	54	28
12	220	91	—	190	50	—	138	27	—	159	53	—	124	32	—	203	73	—
13	128	56	—	153	65	—	97	27	—	94	31	—	87	39	—	130	54	—
14	121	54	—	150	70	—	66	7	—	66	14	—	116	54	—	129	50	—
15	120	51	—	122	35	—	98	19	—	65	17	—	96	30	—	176	76	—
16	85	25	—	209	35	—	90	5	—	90	16	—	134	70	—	150	70	—
17	110	46	—	163	50	—	101	30	—	98	30	—	123	60	—	170	84	—
18	78	28	—	119	45	—	84	13	—	46	22	—	161	62	—	107	38	—
19	153	61	—	217	62	—	91	21	—	78	38	—	177	92	—	101	33	—
20	159	78	—	195	53	—	76	7	—	36	20	—	154	79	—	146	51	—
23	92	42	—	114	14	—	47	10	—	12	1	—	95	41	—	93	40	—
24	98	40	—	129	15	—	32	1	—	29	2	—	99	40	—	113	50	—
Total	3361	1331		3223	963	287	2752	638	320	2237	621	261	2589	1056	27	2841	1083	23
Percentage	40			30—			23+			28+			41—			38+		

The percentage of eggs that hatched in this experiment is not altogether reliable, due to unfavorable conditions which arose. Previous to this time the flies had hatched normally and I still believe that the foregoing experiments measure the absolute fertility of the different stocks and combinations made, within a range of 3 per cent. In this experiment, however, the food became very sour and the flies did little more than crawl out of the pupae and die. The dead animals were searched for and counted. The percentages as given in this table are in consequence too low and are to be taken relatively to each other and should not be compared with the percentages as given in the other tables. Tables 19 and 20 give the details of a second and third repetition of this experiment. The results express more accurately the facts in regard to fertility in these combinations. The plan of the third experiment, table 20, was modified somewhat for it seemed that fertility could be determined fairly accurately by isolating the eggs from mass cultures provided the experiment were controlled by a stock the fertility of which was known. Accordingly hybrids between the truncates whose fertility was low and the Woods Hole stock whose fertility was high were produced and a number of these hybrids were tested in mass culture. The hybrids were also back crossed into the truncates as shown in table 20. In each case a number (the actual number is indicated in the table) of virgin females was placed in a bottle and twice as many males from the stock, in which the combination was desired, added. The males and females were kept separate for four or five days before they were placed together.

At the time of placing the two sexes together an epidemic of mating took place. In each of the bottles about half the pairs were to be seen mating at the same time. None of the eggs were isolated until five days later in order to give all the females a chance to mate and in order to avoid the first few eggs laid, which as I have shown, are not likely to hatch, especially in the case of the truncates. The eggs were isolated day by day in the usual manner. Table 20 gives the result of this experiment. It will be noticed that it is in close agreement with the second experiment of this series.

TABLE 10

Showing the combinations made between the hybrids and the truncates; the number of eggs isolated and the corresponding number of eggs that hatched

T ♀ × B ♂				
NO.	NO. DAYS COUNTED	NO. EGGS ISOLATED	NO. FLIES HATCHED	EGGS NOT INCLUDED
13	2	0	0	0
14	4	35	19	14
15	13	200	69	0
16	2	0	0	0
17	3	40	14	0
18	3	24	8	0
19	4	39	20	0
20	4	45	8	2
21	6	20	7	0
22	14	104	44	12
23	14	31	10	25
24	13	144	38	0
25	9	125	48	5
26	7	120	39	11
27	14	0	0	9
28	20	0	0	0
Total		927	324	78

35 per cent

B ♀ × B ♂				
NO.	NO. DAYS COUNTED	NO. EGGS ISOLATED	NO. FLIES HATCHED	EGGS NOT INCLUDED
6	2	28	22	0
7	16	413	220	0
8	3	75	52	0
9	14	257	165	0
10	6	230	129	0
11	5	134	72	0
12	20	437	277	0
Total		1574	937	0

59.6 per cent

A ♀ × A ♂				
NO.	NO. DAYS COUNTED	NO. EGGS ISOLATED	NO. FLIES HATCHED	EGGS NOT INCLUDED
1	20	460	278	0
2	20	392	164	0
3	5	144	79	0
4	3	57	30	0
5	20	382	257	0
Total		1435	808	0

56.3 per cent

A ♀ × T ♂				
NO.	NO. DAYS COUNTED	NO. EGGS ISOLATED	NO. FLIES HATCHED	EGGS NOT INCLUDED
39	18	284	122	0
40	13	218	107	0
41	20	585	368	0
42	17	414	287	0
43	20	353	210	0
44	20	296	125	136
45	20	283	103	0
Total		2433	1322	136

54.3 per cent

B ♀ × T ♂				
NO.	NO. DAYS COUNTED	NO. EGGS ISOLATED	NO. FLIES HATCHED	EGGS NOT INCLUDED
46	20	513	321	0
47	11	296	158	0
48	20	400	209	11
49	20	481	179	13
50	20	481	232	0
Total		2171	1099	24

50.6 per cent

T ♀ × A ♂				
NO.	NO. DAYS COUNTED	NO. EGGS ISOLATED	NO. FLIES HATCHED	EGGS NOT INCLUDED
29	20	276	150	0
30	8	110	41	0
31	7	103	39	0
32	13	16	5	25
33	3	55	22	0
34	17	117	51	0
35	3	0	0	0
36	10	50	10	0
37	20	141	64	0
38	20	181	76	0
Total		1049	458	25

43.4 per cent

Diagram F expresses the essential relation of fertility as brought out in these three experiments. I shall consider the results of the last two experiments in making up the average since it is evident that these numbers express more nearly the actual facts of the experiment.

TABLE 20

Back crosses between hybrids (of Woods Hole and truncate stock) and the truncate stock. The table gives the number of females that entered into each cross. The numbers on the left side of each column show the number of eggs isolated, the corresponding number on the right the number of flies that emerged.

CROSS	A ♀ × T ♂	B ♀ × T ♂	T ♀ × A ♂	T ♀ × B ♂	B ♀ × B ♂	A	
NO. FEMALES	12	15	28	28	15	15	15
	eggs adults	eggs adults	eggs adults	eggs adults	eggs adults	eggs adults	eggs adults
Jan. 23 '13	110-45	120-65	150-39	61-7	217-130	180-111	225-130
24	60-20	114-54	70-26	43-13	100-0*	103-7*	95-45
25	161-89	130-63	74-7*	55-18	131-72	160-43*	116-72
26	135-41	142-82	120-39	150-56	151-87	115-47	150-97
27	103-47	90-29	133-21	170-40	151-100	150-48	140-91
28	105-39	150-55	200-86	160-74	165-104	166-73	175-144
29	160-80	200-119	175-63	146-42	190-145	231-153	176-123
30	110-33	88-73	125-58	107-46	112-86	203-135	120-81
31	45-25	63-44	61-49	51-16	70-50	100-65	95-67
Feb. 1	71-35	106-55	94-40	45-11	61-45	125-94	100-75
2	90-53	86-49	80-52	30-10	110-80	125-89	36-29
3	40-28	24-14	33-18	20-6	59-41	42-29	25-17
4	38-20	45-29	53-27	43-19	35-25	60-39	44-31
5	20-8	45-22	50-18	10-3	35-25	21-12	30-21
6	43-23	50-24	34-18	20-7	35-31	86-59	
7	27-12	78-37	45-15	30-8	100-62	130-58	98-76
8	39-16	91-44	34-12	27-8	102-55	120-55	50-38
9	52-25	150-75	105-40	43-12	53-34	100-49	100-74
10	160-88	85-49	100-34	28-0*	121-63	150-80	66-50
11	140-73	114-42	102-36	17-1	115-70	115-60	135-81
13	50-33	51-22	50-20	0	55-28	53-38	54-36
14	51-26	50-18	50-17	0	55-30	56-30	55-43
Total.....	1810-859	2072-1064	1864-728	1228-397	2122-1363	2328-1324	2085-1421
Per cent...	47	51	39	32	64	57	70

* These bottles met with an accident and are not considered in the totals.

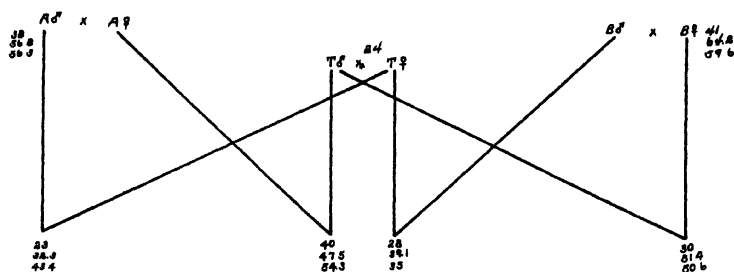


Diagram F Summary of tables 18, 19 and 20, showing the effect on fertility of back-crossing the hybrids into the recessive low-producing truncates.

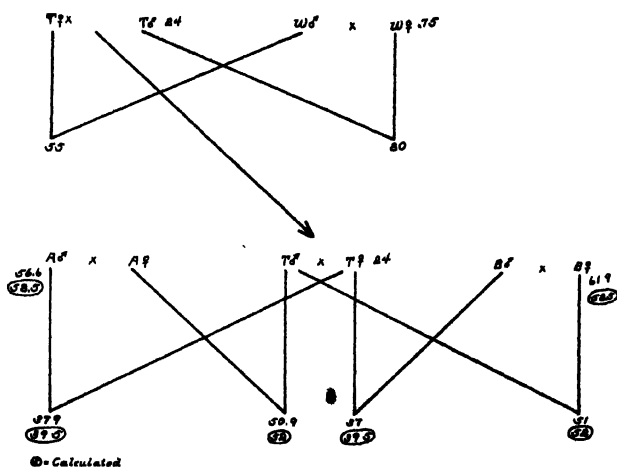


Diagram G Expresses the essential relations of the behavior of fertility in heredity as based on the foregoing experiments. The numbers in the circle express the calculated percentage. The other numbers express the observed percentage of fertility.

In diagram G a composite picture is given of all the experiments that bear on fertility in these crosses where an accurate method of measurement has been employed.

If 24, 75, 55, and 80 express the actual fertilizing powers of the gametes of the different combinations then the percentages as given in the hybrid back crosses can be explained on the following assumption.

The hybrid A is made up of T ♀ and W ♂. Let us assume that its germ cells segregate into the parental types in so far as factors for fertility are concerned. Accordingly A ♀ produces two kinds of eggs, T eggs and W eggs. A ♂ will produce two kinds of sperm, T sperm and W sperm. Diagram H expresses the relation as to what happens when the gametes are brought together.

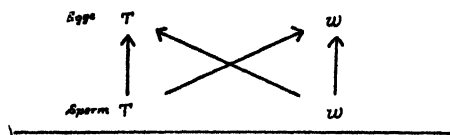


Diagram H Showing the possible combinations of the gametes of the hybrids (A) on the assumption that the germ cells have segregated into the parental types in respect to factors for fertility.

- When a T sperm fertilizes a T egg 24 per cent of the eggs hatch
- When a T sperm fertilizes a W egg 80 per cent of the eggs hatch
- When a W sperm fertilizes a T egg 55 per cent of the eggs hatch
- When a W sperm fertilizes a W egg 75 per cent of the eggs hatch

The sum, 234, divided by 4 gives on this assumption the actual fertilizing power of the combination, or 58.5, which corresponds very closely with the 56.6 observed. In like manner the other combinations may be determined. I have placed in a circle in each case the calculated percentage of fertility; the number outside the circle expresses the observed percentage. The agreement between the two sets of numbers lends support to the view that the germ cells of the hybrid do segregate into factors identical with those of the parents in so far as factors that are responsible for fertility are concerned.

We find here, I believe, the key for the low fertility of the truncates. They have become homozygous for many factors. The corresponding rise in fertility when crossed into other races is to be explained on the assumption that the combination formed is heterozygous for more characters upon which fertility depends and consequently more likely to develop. The assumption seems a reasonable one to make in light of the fact that we are able to predict the fertilizing power of the gametes in a given combination.

It will be recalled that over sixty generations of continuous selection has failed to produce pure truncate stock. May not this result be explained on the grounds that only the heterozygous forms with respect to this structure ever reach maturity; the homozygous or pure truncate animals never come into existence? It is of interest in this connection to recall Cuénot's failure to produce yellow mice that would breed true.

The reduced fertility of the inbred stock in successive generations of inbreeding may also find its explanation on the assumption that the gametes have reduced to a condition approaching homozygosity for as I was able to show in the F_{14} generation a larger percentage of zygotes developed as a result of outbreeding.

FERTILITY AND HIGH PRODUCTIVITY OF THE HYBRIDS

It has been pointed out that the hybrids that resulted from the crosses between the low-producing truncates and the high-producing wild stocks give rise to a large number of offspring. They produce on an average many more young than their high-producing parents. The fertilizing power of their gametes when inbred was only 56.6 per cent, while in the Woods Hole stock it was 75 per cent. In other words, the productivity of the hybrids is higher than its high-producing parent, but the fertilizing power of its gametes is lower.

This apparent contradiction is accounted for by the fact that the output of eggs of the hybrid female is greatly increased and despite the fact that the fertilizing powers of the egg and sperm when placed together is relatively low, nevertheless the production of offspring is very high. This fact shows how easily one might be misled by using the number of offspring as a measure of the fertilizing power of the egg and sperm when not controlled by more accurate means. A comparison of the number of eggs laid by the hybrid, table 21, with the number laid by the Woods Hole females, tables 14 a, 15 a and 15 d will show that the output of eggs from the hybrid is practically twice that of its high-producing parent.

The following controlled experiment, table 22, confirms the foregoing conclusion and also shows that it makes no difference

in regard to egg production whether the father or the mother of the hybrid came from the low-producing truncate stock.

In this experiment conditions were as uniform as possible. The virgin females were in each case paired with three of their brothers. The eggs were counted each day with the aid of a dissecting microscope and discarded. The females in this experiment were given a chance to lay their eggs on dried apples that had been soaked in water and allowed to become slightly sour. This insures great accuracy in counting, as the white eggs are easily seen against a brown background.

The count as given in tables 21 and 22 supports the conclusion that the hybrid female has a greatly increased output of eggs as compared with the Woods Hole female. Now despite the fact that only about 56 per cent of her eggs hatch when tested with her own male, yet she is able to produce as many if not more offspring than her high producing parent, the fertility of which is 75 per cent. We should expect the hybrid to lay approximately twice as many eggs. An analysis of table 22 shows this to be true. It is true that no far-reaching conclusion can be based on such a round-about treatment as this, taken alone. This evidence must be taken in connection with the other evidence presented. If the percentage evidence as given is correct, and if it is true that the hybrids are excellent producers, as judged by the number of offspring produced, then we should expect the egg-production to be greatly increased. The count as given here verifies the expectation and makes the former conclusions more certain.

The anomalous condition that the fertility of this animal may be relatively low and yet its productivity high, finds its explanation in the facts presented and shows, as before stated, how one may easily be misled in regard to fertility in heredity by using the number of offspring produced as a guide, when not checked up by more accurate means.

The increased egg-laying capacity on the part of the female may seem strange at first sight, especially when we recall the fact that neither parent possessed it to such a marked degree. But let it be recalled that the hybrid is more virile, as judged by the length of life, than either parent and that the same thing is probably true in regard to their reaction to light. In fact, in every way these

TABLE 21

Number of eggs laid by the offspring of the crosses between the low-producing truncates and the high-producing Woods Hole stock. These are the same females given in table 18

(A) A ♀ × A ♂			(B) B ♀ × B ♂		
NO.	DAYS COUNTED	NO. EGGS LAID	NO.	DAYS COUNTED	NO. EGGS LAID
1	34	1056	10	33	974
2*	6	144	11*	10	221
3	14	0	12	46	826
4	13	372	13	84	1807
5*	7	170	14	48	1429
6*	4	116	15*	15	351
7*	15	462	16*	28	782
8	88	2184	50	26	552
9	71	1613	51	33	756
56*	30	828	52	52	601
57*	16	371	53	44	652
58	52	1246	54	48	901
59*	14	304	55*	9	200
60*	59	1265	Total	476	10052
61	48	1360			
Total	471	11491	Average per day	21.12	

Average per day 24.4

* Escaped

TABLE 22

Showing the total output of eggs of the Woods Hole stock and their hybrid children when crossed to the truncate stock

WH ♀ × WH ♂			A ♀ × A ♂			B ♀ × B ♂		
NO.	LIFE OF ♀	NO. EGGS LAID	NO.	LIFE OF ♀	NO. EGGS LAID	NO.	LIFE OF ♀	NO. EGGS LAID
13	41	812	1	51	1406	7	35	835
14	51	443	2	14	403	8	38	1043
15	61	9	3	34	886	9	55	1511
16	61	896	4	31	589	10	29	740
17	61	369	5	52	1482	11	50	1204
18	16	346	6	52	232	12	25	490
19	54	776	Total....	234	5998	Total....	232	5823
20	57	1031						
21	54	1000						
22	61	1034						
Total..	517	6216						

Average number of eggs laid per day 12.02.

Average number of eggs laid per day, not considering number 15, 13.61.

Average number of eggs laid per day 25.63.

Average number of eggs laid per day 25.09.

animals seem to be better somatically than either parent. To be sure, their fertility inter se is much lower but it should be remembered that while the individuals belong to one generation the egg and sperm produced belong to the next.

The following suggestion is offered as a tentative explanation: It is probable that some factor or factors, let us say, regulate egg production in the normal wild fly.¹ Let us designate these as AB.

Let us assume that the truncates have dropped out some factor from this mechanism, then their formula would be expressed by Ab. The other stock used had been inbreeding in mass cultures in the laboratory and had probably lost some factor, let us say A. Then their formulae would be expressed by aB. When the two races are crossed, the two factors are brought together again, thus ABab, and the normal egg-laying function is restored. The explanation holds at least tentatively for other improvements to be seen in the hybrid. The number of factors concerned is not to be looked upon to be as simple as the formula would seem to indicate. Instead of two factors, as the formula shows, there may be many units concerned, but the principle is the same. The things lost out of the germ-plasm of one are made good by those present in the other and thus normal conditions are restored. If this is so we should expect to find members of this species in nature that live well on to one hundred days and some that have high egg production and high fertility combined. They should produce well on to two thousand flies.

FERTILITY OF THE LONG-WINGED BROTHERS AND SISTERS OF THE TRUNCATES

When the long-winged flies thrown by the truncates are bred together their productivity is greater than their truncate brothers and sisters. This fact is brought out in table 23 where 120 is recorded as the average per pair. Their truncate brothers and sisters used for control produced an average of 77 offspring per pair. The flies were of the same ancestry and subject to the same environmental conditions. That the long-winged forms produce

¹ Whether the actual formation of germ cells, in this case eggs, or the regulatory mechanism that governs their actual maturation and discharge is involved is a problem more difficult of solution.

more offspring than their truncate brothers and sisters is borne out by table 24, in which the F_2 generation of the longs as thrown by the truncates are tested in pairs. In this experiment they gave an average of 92 offspring per pair, while 34 pairs of truncates used for control gave an average of 47 offspring per pair. (Table 1, F_{45} .)

I have pointed out that the number of offspring produced by a pair of flies is not an infallible guide to the fertilizing power of their gametes. That the fertilizing power of the egg and sperm of the long-winged forms is greater than that of the truncate flies is supported by the following experiment, in which the eggs were isolated and 37 per cent hatched. Since I have never been able to obtain more than 24 per cent of fertile eggs from the truncates I conclude that this difference when taken in connection with the other evidence is significant. Attention is called to No. 31, table 14b, in which the fertility of a long-winged female is 38 per cent, or 14 per cent greater than her truncate sisters.

The data recorded in table 25 are too small upon which to base any very definite conclusion in regard to this obscure phenomenon,

TABLE 23

This table shows the result of testing in pairs the long-winged brothers and sisters of the truncates. For testing of truncate brothers and sisters to these, see table 1.— F_{44} .

NO.	TRUNCATE ♂	TRUNCATE ♀	TOTAL TRUNCATES	LONG ♂	LONG ♀	TOTAL LONGS	GRAND TOTAL
1	5	5	10	41	39	80	90
2	15	14	29	76	75	151	180
3	2	4	6	21	23	44	50
4	3	4	7	67	75	142	149
5	11	6	17	37	28	65	82
6*	—	—	—	—	—	—	—
7	12	17	29	53	59	112	141
8	7	7	14	56	56	112	126
9*	—	—	—	—	—	—	—
10	15	10	25	70	49	119	144
Total.....	70	67	137	421	404	825	962
Average...	—	—	—	—	—	—	120

* Sterile.

TABLE 24

Showing the result of breeding in pairs the long-winged individuals selected from the previous experiment, the results of which are shown in table 23.

ANCESTRY NO.	SERIAL NO.	TRUN- CATE ♂	TRUN- ♀	TOTAL TRUNCATE	LONG ♂	LONG ♀	TOTAL LONGS	GRAND TOTAL
2	1	13	7	20	66	49	115	135
2	2	0	0	0	33	33	66	66
2	3	0	0	0	6	7	13	13
2	4*	—	—	—	—	—	—	—
2	5	2	5	7	8	7	15	22
2	6	0	0	0	68	42	110	110
3	7*	—	—	—	—	—	—	—
3	8	5	4	9	57	53	110	119
3	9	3	2	5	37	35	72	77
4	10	7	2	9	42	56	98	107
4	11*	—	—	—	—	—	—	—
4	12	6	8	14	59	77	136	150
4	13	0	1	1	27	42	69	70
4	14	0	1	1	41	38	79	80
4	15	3	1	4	24	27	51	55
4	16	13	15	28	72	49	121	149
4	17	15	10	25	66	53	119	144
4	18	1	4	5	8	5	13	18
8	19*	—	—	—	—	—	—	—
8	20	2	4	6	17	31	48	54
8	21	4	11	15	57	40	97	112
8	22	0	1	1	4	5	9	10
10	23	5	9	14	86	86	172	186
10	24	24	45	69	50	52	102	171
10	25*	—	—	—	—	—	—	—
10	26	1	4	5	30	41	71	76
Total....		104	134	238	858	828	1686	1924
Average..		—	—	—	—	—	—	92

* Sterile

TABLE 25

Showing the number of eggs isolated and the corresponding number that hatched on pairing the long-winged males and females thrown by the truncates

NO.	NO. EGGS ISOLATED	NO. FLIES HATCHED	NO. EGGS NOT INCLUDED
1	152	66	0
2	41	14	31
3	71	20	31
4	73	22	20
5	45	18	0
Total	382	140	82

but that the productivity of the long-winged individuals is greater than their truncate brothers and sisters I can testify from my experience with them in mass culture, and it would seem as though this increased productivity is due to the increased fertilizing power of the egg and sperm. I shall return to this question in Part IV.

BEHAVIOR OF THE TRUNCATE WING IN HEREDITY

Attention has already been called to the fact that many generations of inbreeding the truncate brothers and sisters has failed to purify this stock. The truncate always throw long-winged males and females. The long-winged flies in turn throw some truncates. Table 1 gives the number of truncates and longs that appeared in the different generations by continually selecting the truncates. From a total of 6356 there are 5469 flies with truncate wings and 887 flies with long wings; a ratio of 1 long to 6.2 truncate. During the period under investigation this ratio remained fairly constant.

When the long-winged flies thrown by the truncates were bred together there appeared in a total of 962 flies, 825 with long wings and 137 with truncate wings; a ratio of 6.2 longs to 1 truncate. When the F_2 generation of long-winged flies were bred together the different classes appear as shown in table 24. In a total of 1924 there are 1686 long-winged flies and 238 truncate flies. The males and females are about in equal proportions. This gives a ratio of 1 truncate to 7.1 long. This ratio is practically the opposite of the condition found in the truncate wing which threw 1 long to 6.2 truncates.

When the truncates are paired with the long winged wild stocks such as the inbred or Woods Hole, long wings are dominant to short, but not completely so; for while no truncate wings appear a new class of wing arises. This is a long wing with small crescent shaped piece cut out of the inner margin at the tip of each wing. These will be referred to as 'crescents.'

In the cross as given in table 3 there appeared among a total of 1784 hybrids, 41 crescent males and 35 crescent females, a ratio of 1 crescent to 21.2 longs. In the reciprocal cross from a total of 4711 offspring there were 30 crescent males and 84 crescent

females; a ratio of 1 crescent to 40.3 longs. I shall neglect this ratio in further discussion as it appears to be aberrant.

In the cross as given in table 6 a, there were 34 crescent males and 19 crescent females, a ratio of 53 crescent to 1127 long or 1 to 21.3. In the reciprocal cross, table 6b, there were 30 crescent males and 49 crescent females in a total of 1714. This gives a ratio of 79 crescent to 1635 long or 1 to 20.7.

The different classes appeared in F_2 generation of the crosses as follows. From a total of 16096 as given in the cross in table 9 there are 516 truncate males, 778 truncate females, 431 crescent males, 633 crescent females, 6881 long males and 6857 long females. In the reciprocal cross there are 273 truncate males, 332 truncate females, 315 crescent males and 436 crescent females, 4986 long males and 5458 long females. Out of a total of 27896 there are 1903 truncates and 1881 crescents. This gives a ratio of 1 truncate to 13.2 longs and 1 crescent to 13.4 longs. The ratio is practically the same in both the cross and the reciprocal. To summarize in a general way, we may say that during the period of investigation the truncates threw one long in $7\pm$ truncates. Their long-winged brothers and sisters threw 1 truncate in $7\pm$ longs. The new type of wing, the crescent, appeared in the crosses between the truncates and the wild stocks and in the ratio of 1 crescent to 21 longs in both the cross and its reciprocal. In the F_2 generation crescents and truncates appear in equal numbers from both the cross and the reciprocal and in the ratio of 1 to 14.

These ratios apparently do not fall under any Mendelian explanation and yet I believe that any explanation of these ratios must take into consideration the great viability of the truncate stock and my opinion is that when more facts are available this case may be found to fall under a Mendelian formula because there is evidence of segregation and because definite ratios appear according to the combinations that are made.

SEX RATIO

It is not the purpose here to enter into a discussion of sex-determination. I merely wish to bring together the data from the foregoing experiments that bear on the question of sex-ratios. Since the viability has been so great in some of my strains it seems remarkable that disturbances in the sex-ratio have not been encountered. The tables show the ratios to be remarkably constant, with a slight excess of females in almost all cases. The females emerge first and if a count is made at this time there is in almost all cases a large excess of females. At times the ratio may be as high as two or three females to one male. Toward the middle of the count, however, the ratio approaches equality and toward the end of the count the males are usually in excess and this tends to equalize the sex ratio. It is not that the first eggs laid produce females but that the egg which is to develop into a female carries its development through on an average from twelve to twenty-four hours more rapidly than the male. This is evident from the hundreds of bottles from which my counts have been made.

Different strains of these flies vary somewhat in their rate of development. The truncates always emerged from two to four days later than the wild stocks used in control. The hybrid fly carries its development through to the hatching stage more rapidly than either of the parent strains.

I have said that the sex-ratio is remarkably constant, with the females slightly in excess. This statement applies to the inbred stock, the truncate stock and the Woods Hole stock when brothers and sisters are paired. Diagram 1 shows the ratio of males to females to be 100 to 103; 100 to 103 and 100 to 107 respectively. I think that the slight excess of females is due to the factors to which reference has already been made. When the different races are crossed into each other a rather large excess of females appears. This statement also holds for the children and grandchildren when tested together in pairs. I do not believe that the early emergence of the female from the pupae will ac-

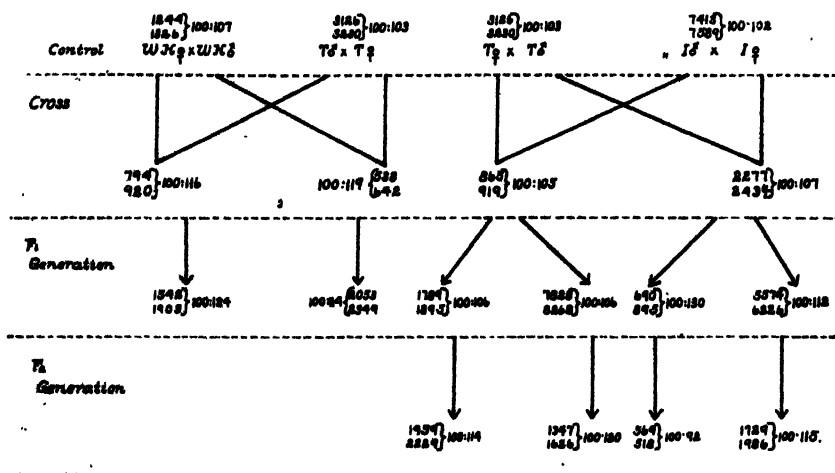


Diagram I Showing the ratio of males to females. Compiled from the foregoing experiments. T = truncate; WH = Woods Hole; I = inbred.

count for the rather large excess of females in these crosses. It should be stated here as a matter of fact that the sex ratio does not change with the age of the female. Diagram I gives the sex-ratio as compiled from the foregoing experiments.

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A QUANTITATIVE DETERMINATION OF THE ORIENT- ING REACTION OF THE BLOWFLY LARVA (CALLIPHORA ERYTHROCEPHALA MEIGEN)

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TWENTY-FOUR FIGURES

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STATEMENT OF THE PROBLEM

In the work done thus far in animal behavior, no successful attempt has been made to apply any form of precise measurement to movements produced by light of known differences in intensity. The experiments on the blowfly larva described in this paper were devised for the purpose of making exact quan-

titative measurement of the light reactions in some suitable and easily available animal. The light was applied as two opposed beams, the intensity of which could be easily controlled and precisely measured. The responses to the stimulation produced by two beams of different intensity acting simultaneously on opposite sides of the same animal were measured in angular deflections from an initial path of locomotion.

LITERATURE

The earliest paper on the light reactions of blowfly larvae was that of Pouchet, in 1872. He described a series of experiments with daylight and artificial light, showing the negative character of the light response of the larvae of several species of the old Linnaean family of Muscidae. A considerable portion of the paper is devoted to experiments devised to locate the light recipient organs. The fact that the light response did not disappear when the sensory cones of the anterior end were excised, led Pouchet to conclude that the imaginal discs of the adult compound eyes were the sensitive organs. This conclusion was borne out by his observations on the increase of sensitiveness with the age of the larva, which coincides with the increase in development of the imaginal discs. The following quotation sets forth his interpretation of the way in which the imaginal discs serve, not only to perceive the light but also the direction of the rays (1872, p. 316): "La lumière, frappant, sous des angles différent, les surfaces toutes différent inclinées sur l'horizon des yeux embryonnaires, donne à l'animal le sentiment de la direction des rayons, par l'intensité relative avec laquelle ils affectent, grâce à leur incidence, les différent yeux."

This conclusion is interesting in its foreshadowing of the question of the relative effect of 'ray direction' and 'intensity difference' on orientation which grew out of the tropism controversy nearly twenty years later.

The work of Pouchet, like most studies of behavior of this period, was done on the basis of preference as shown by experimenting. Conditions were so arranged that the animals could move into regions of higher or lower intensity or of particular

color, and the results were interpreted in terms of human experience. The behavior of an animal that was assumed to have the freedom of choice was said to indicate its preference.

The students of plant behavior—because the subjects of their experiments were remote from human activities—had begun to free themselves from the anthropomorphic point of view long before the zoölogists. With their earlier analytical outlook on the natural phenomena, came an earlier seeking for mechanical explanations. In the middle* of the last century, when animals were still going toward light “because they liked it,” or “because it aroused their curiosity,” the botanists were seeking to determine the nature of the mechanism involved in the reactions of plants to light.

As early as 1831 de Candolle had used the term heliotropism to describe the bending of plants toward the sun. This he believed to be due to the direct effect of local difference of intensity, growth being retarded on the more highly illuminated side. The inadequacy of this explanation was shown by Charles Darwin and his son Francis in their work on “The power of movement in plants;” these investigators demonstrated the difference between sensitive and reacting tissues and the transmission of stimuli from one to the other.

Sachs, however, did not accept the theories of the Darwins and advanced the explanation that the turning was controlled by the direction in which the rays of light penetrated the tissues of the plant. The simplicity of the theory of Sachs was very alluring, but, like many “simple mechanical explanations,” it fitted only a few of the facts.

Soon after the work of Sachs appeared, Loeb took up the study of animal reactions with the purpose of analyzing the phenomena in terms of physics and chemistry in opposition to the anthropomorphic ‘explanations’ prevalent at that time among zoölogists. His attitude is well expressed by the following quotation from one of his later works. He says ('05, p. ix) “I consider a complete knowledge and control of these agencies (which determine behavior) the biological solution of the metaphysical problem of animal instinct and will.” Whether or not future work bears out

all Loeb's conclusions is a matter of small importance beside the tremendous advance in clear thinking which has resulted from the analytical attitude he has from the first maintained.

In his earliest work bearing on the orientation of animals to light ('88), he cites the reactions of fly larvae in support of his explanation of orientation. The main interest of this work was theoretical and but few facts were added to the fairly complete account already published by Pouchet. Perhaps the most significant of these were his establishment of the restriction of the sensitive region to the anterior end of the larva and the balanced reaction to equal lights acting on opposite sides of the body. Loeb concluded that the factor of prime importance in orientation was the direction in which the rays of light penetrated the tissue, as Sachs had believed for plants. This view, however, he has since abandoned, as may be seen from the following quotation ('06, p. 130),

We started with the assumption that the heliotropic reactions are caused by a chemical effect of light; in all such reactions, time plays a rôle. We assume, furthermore, that if light strikes two sides of a symmetrical organism with unequal intensity, the velocity or the character of the chemical reactions in the photosensitive elements of both sides of the body is different; that in consequence of this difference the muscles, or contractile elements on one side of the organism are in a higher state of tension than their antagonists. The consequence is a curvature or bending of the head. This is followed by a turning of the body, kept up until the stimulus acts equally on the bilaterally located sensitive areas. When such a condition of balance is attained, the animal no longer deviates toward either side, but pursues a direct path toward or away from the source of light.

If it be true that the immediate effect of light in causing the heliotropic reactions is of a chemical nature, we should expect that it must be possible by use of chemicals to control the precision and sense of heliotropic reactions ('06, p. 131).

The striking results which Loeb ('93, '04, '06) obtained in his experiments on the chemical control of heliotropism are the strongest sort of evidence for his interpretation that light reactions depend fundamentally on a chemical reaction, the extent of which is dependent on the intensity of the light.

Loeb thus opened a new field of tremendous interest. The mechanical explanation of orientation to light became the ob-

jective point of work on light reactions. Interest no longer centered about the cataloguing of the positive or negative response of various forms, but about the developing of a general theory of light reactions.

With the possible exception of Engelmann's work on swarm spores, most of the work on behavior up to this time had dealt with mass reactions rather than with the details of individual reactions. In 1897 Jennings began a study of the aggregations so commonly found in the cultures of *Paramecium*, by observing in the minutest detail the reactions that brought a single individual into the aggregation and held it there. The work on *Paramecium* was followed by many other papers on the Protozoa and some on the lower Metazoa, all characterized by the same thoroughness in the observation of individual behavior. Such methods brought out many facts of great interest, previously overlooked.

One of the points on which Jennings lays great emphasis is that behavior does not depend on the external stimulus alone. He believes that there are various internal factors which modify the reactions to the same external conditions. Former stimuli and the reactions of the organisms to them, as well as the metabolic processes constantly going on within the animal, have their effect on the physiological state. The physiological state in turn determines to a large degree the reaction of the organism to external stimuli.

Jennings also found that in the Protozoa on which he worked there were no bilaterally located sensory areas and that the position of orientation was not one in which the median plane of structural symmetry was placed in a definite position with reference to the source of stimulation. The 'tropism theory,' as put forward by Loeb, evidently did not apply to these organisms. Jennings observed that changes in the direction of locomotion were brought about by 'motor reflexes' directed toward a structurally definite side of the organism. He characterizes this method of orientation as one of 'trial and error.' The 'varied movements' of locomotion involve contact with varying environmental conditions, selection from among these conditions is

brought about by the 'motor reflex' produced whenever a stimulus is encountered. This usually removes the animal from the source of stimulation, for the 'structurally definite side' toward which the reflex is directed is that opposite to the side on which the sensitive area is located. If the distribution of the stimulus in space is such that these reflexes hold the animal on a direct path with reference to the source of stimulation, a response resembling a 'tropism' results, but the method of its accomplishment is different from that assumed by Loeb. Jennings did not, however, believe that the orientation of all animals was brought about by the motor reflex ('06, p. 271). "In the symmetrical Metazoa we of course find many cases in which the animal turns directly toward or away from a source of stimulation without anything in the nature of preliminary trial movements."

About this time Holmes ('05) published a detailed account of the movements involved in the orientation of the individual blowfly larva. He lays great stress on the significance of the side to side swinging of the anterior end which occurs when the larva is suddenly stimulated by lateral illumination. These random movements, he believes, afford a means of selecting a favorable direction of advance. He says ('05, p. 105):

There is, so far as I can discover, no forced orientation brought about by the unequal stimulation of the two sides of the body, but an orientation is produced indirectly by following up those chance movements which bring respite from the stimulus. I do not deny that there may be an orientating tendency of the usual kind, but if there is, it plays only a subordinate rôle in directing the movements of the animal. The orientation of these forms is essentially a selection of favorable chance variations of action and following them up (p. 106). It may be said to be a form of the trial and error method minus the element of learning by experience.

Hermes has published two articles on the light reactions of the larvae and adults of the Sarcophagid¹ flies. The earlier paper ('07) is based largely on a field study of the light reactions as they affect food habits and migration. The second paper ('11) contains an account of numerous laboratory experiments in which he has brought out a number of interesting points, such as the increased rate of crawling under increasing intensity of illumi-

¹ Hermes bases his classification on Girchner's system, published in 1896.

nation, the positive reaction of aggregations of feeding larvae to a light of moderate intensity, and the circus movements performed by larvae with one side of the anterior end blackened. A valuable part of the paper is occupied with the confirmation by more exact experimental data, of several of the earlier conclusions.

Mast in his extensive treatise on "Light and the behavior of organisms" has collected practically all the important work of the earlier writers and added many experiments of his own. His original work is similar to that of Jennings in the careful observation of the details of individual behavior. One cannot fail to be impressed by the painstaking methods of his experiments, but it is difficult to see how his results invalidate the theories of Loeb so completely as he would have us believe.

Considerable space is devoted to the reactions of fly larvae. Experiments are described which bear out the conclusion of Loeb and Herms that the sensitive region is restricted to the anterior end, but none of them aid materially a precise localization of the sensitive organs. His experiments on the effect of intensity on the rate of locomotion failed to yield any very definite results, largely because they were made with horizontal lights and hence the shadow of the animal's body prevented the direct operation of the light on the sensitive anterior end. The most interesting part of his work is the series of experiments by which he establishes that, in this form as well as others on which he worked, orientation depends primarily on the intensity which operates on the sensitive surfaces, but depends on 'ray direction' only in so far as it modifies the operative intensity on the receptive areas. His analysis of orientation will be taken up in same detail in the discussion of theories of orientation.

Several authors have experimented with the effect of colored light on maggots, but the work of Gross ('13) is, by reason of the refinements of his apparatus and methods, by far the most accurate that has been done in this field. The colors used in his experiments were practically monochromatic and were accurately measured for intensity by means of a radiomicrometer. The sequence of effectiveness which he established for the larvae—green, blue, yellow, red, decreasing in the order named—is unusual in the greater effectiveness of the green than the blue.

It is interesting that in the imago the conditions are reversed, and the blue is more effective than the green.

In the work which has been done on the light reactions of the blowfly larva there is essential agreement as to the general manner in which the animal actually behaves under various experimental conditions. The interest of recent work has centered about the discussion of details of behavior which have been adduced in support of one or the other of the theories of orientation. There has been no attempt made to obtain definite measurements of any phase of the orienting reaction. The purpose of the present paper is to devise a method of quantitative measurement which shall be available in work on light reactions and to apply such a method to the orienting reaction of the blowfly larva.

APPARATUS

The apparatus used in these experiments was constructed so that the opposite sides of the animal under observation could be subjected to opposed beams of light the actual and relative intensity of which could be varied at will. The plan of the apparatus is shown in figure 1.

It consisted of a horizontal wooden frame in the form of an isosceles right triangle. As shown by the dotted lines on the diagram, this frame was so proportioned that a similar triangle measuring two meters on the base and 1.414 meters on the equal legs, could be laid out on it. Where the side bars of the frame came together to form a right angle, a horizontal platform 65 by 35 cm. was attached. On this platform, at the apex of the triangle were set up five 220-volt Nernst glowers, mounted vertically and about 5 mm. apart, each with a switch in circuit. Their position was such that no glower interfered with the light thrown on the mirrors by any other glower. When less than the five-glower intensity was desired, symmetrically placed glowers were used.

A portion of the light from these glowers passed through the horizontal rectangular apertures (3.2 by 6.1 cm.) in the screens *d* and *d'*, placed 25 cm. from the glowers.

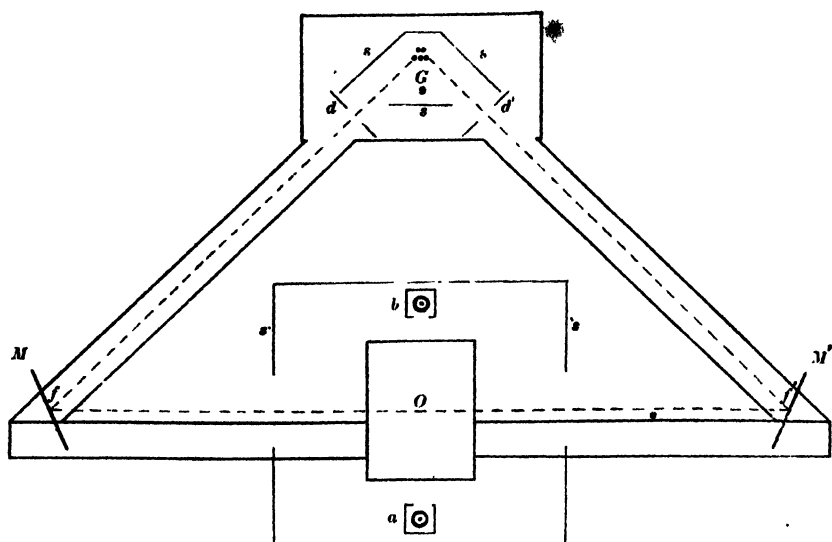


Fig. 1 Diagram of apparatus used to produce differential bilateral light stimulation. *G*, five 220-volt Nernst glowers; *M* and *M'*, mirrors; *f* and *f'* central, point of mirrors; *O*, center of observation stage; dotted lines, central ray of beam of light from the glowers reflected to *O* by the mirrors; *d* and *d'*, screens with rectangular openings; *s* and *s'*, light shields; *a* and *b*, 2 c. p. orienting light with screens.

The central ray of light from the apertures fell on the centers of the vertical mirrors *M* and *M'*, which were cut from the best French glass, and, to insure uniformity, from the same piece. These mirrors were placed at such an angle that they reflected the central ray of the beams *Gf* and *Gf'* to the observation point *O* directly opposite the glowers. It will be seen that the apparatus was so constructed that the courses taken by the light over the two paths from *G* to *O* were equal in length and symmetrical in position. Furthermore, the central rays impinging at *O* were in the same straight line but came from opposite directions. The two beams of light reaching this central point should be of equal intensity, and careful photometer tests showed them to be so. By using a single source of light for both beams, the uncertainty due to the possible fluctuations in two separate sources of light was eliminated.

A horizontal stage of slate 30 by 48 cm., on which the animals were observed, was placed at the same level as the center of the glowers, and in such a position that the observation point *O* fell at its center. At either end of this stage, a 2-candle power incandescent lamp was set up at a distance of 55 cm. from the central point *O*. By means of these 'orienting lights,' (fig. 1 *a* and *b*) a negatively phototactic animal could be started from either end of the observation plate, directly across the field of light from the mirrors. Light screens around the glowers, *s*, and a larger set of screens around the observation stage, *s'*, shut out extraneous light and reduced reflection to a minimum. The orienting lights also were screened except for a small horizontal aperture throwing its central ray to *O*.

With the apparatus thus set up, a maggot could be made to move on to the observation stage, away from one of the orienting lights, so that two equally intense beams of light fell on its opposite sides. By reducing the intensity of one of the beams of light, differential bilateral stimulation could be set up, of any desired proportion or intensity. It was important to find a reliable method of reducing one of the beams of light. The episcotister, though very convenient, has been shown to be an unreliable means for the accurate reduction of light intensity (Parker and Patten '12). Several forms of gratings were tried, none of which gave a perfectly uniform field. The difficulty of securing a uniform field, with diaphragms of small enough aperture to give the desired intensity differences, was very great. The most satisfactory means seemed to be to move the observation stage to points on the basal arm of the apparatus where, according to the law $I \propto \frac{1}{d^2}$, the intensities should be of the desired ratio. Such a method, though cumbersome, gave an absolutely uniform field, and the intensity could be figured with great accuracy.²

² Hyde ('06) has computed very accurately from the law $I \propto \frac{1}{d^2}$, the amount of variation when the source of light is a cylinder of the dimensions of a Nernst glower instead of a point. For the distances used in this apparatus, the variation is from +0.08 per cent to +0.08 per cent.

The percentage differences in the two lights used in this series of experiments were $66\frac{2}{3}$, 50, $33\frac{1}{3}$, 25, $16\frac{2}{3}$, $8\frac{1}{3}$ per cent, and as a control, equality. The points on the basal arm of the apparatus at which these percentage differences exist are: $66\frac{2}{3}$ per cent, 63.9 cm.; 50 per cent, 41.4 cm.; $33\frac{1}{3}$ per cent, 24.6 cm.; 25 per cent, 17.4 cm.; $16\frac{2}{3}$ per cent, 11 cm.; $8\frac{1}{3}$ per cent, 5.4 cm. to the right or left of the central point *O*. The percentage differences are in all cases expressed in terms of the stronger light. The actual intensities of the beams are given in table 1.

With the apparatus set up for unequal opposed lights, negative animals should deflect toward the weaker light, and the deflection should be greater as the difference between the lights is increased. This deflection measured in degrees gives the desired quantitative expression of the physiological effects of the light.

MATERIAL AND METHODS

1. Cultures

The raising of blowfly larvae, and the duration of the various stages of their development under natural and artificial conditions, has been dealt with by Herms ('07, '11). He also made observations on the differences in behavior between certain of

TABLE 1

The intensities in candle meters of all the lights used in the series of experiments.

The four-glower intensity is slightly lower than would be expected because the position of the glowers was such that there was a minimum of mutual heating at this intensity. The $83\frac{1}{3}$ per cent intensities do not fit in with the series because to obtain such a large difference it was necessary to re-set the mirrors.

PER- CENTAGE DIFFER- ENCE IN LIGHTS	RATIO OF LIGHTS	INTENSITY OF WEAKER LIGHT IN CA. METERS					INTENSITY OF STRONGER LIGHT IN CA. METERS				
		1 glower	2 glowers	3 glowers	4 glowers	5 glowers	1 glower	2 glowers	3 glowers	4 glowers	5 glowers
Equality	1 to 1	6.32	13.9	24.6	31.5	41.1	6.32	13.9	24.6	31.5	41.1
$8\frac{1}{3}\%$	11 to 12	6.05	13.4	23.6	30.2	39.3	6.61	14.6	25.7	32.9	42.9
$16\frac{2}{3}\%$	5 to 6	5.77	12.8	22.8	28.8	37.5	6.93	15.3	26.9	34.5	45.0
25 %	3 to 4	5.50	12.1	21.4	27.4	35.7	7.34	16.2	28.5	36.5	47.6
$33\frac{1}{3}\%$	2 to 3	5.21	11.5	20.2	25.9	33.8	7.83	17.1	30.5	39.0	50.9
50 %	1 to 2	4.60	10.2	17.9	22.9	29.9	9.20	20.2	35.8	45.9	59.9
$66\frac{2}{3}\%$	1 to 3			15.4					46.4		
$83\frac{1}{3}\%$	1 to 6			7.6					46.4		
100 %	0 to ∞			0.0					24.6		

the species, a detail which has been too often neglected in work on behavior. Though the species he worked on especially, *Lucilia caesar*, and *Calliphora vomitoria*, reacted essentially in the same manner, the former was noticeably more sensitive to light than the latter. Similarly with various *Planaria*, Walter ('07) has shown well defined species differences in the light response. To avoid inconsistencies which might arise by the neglect of species differences, except for some preliminary experiments, the work was done with the larvae of a single species, *Calliphora erythrocephala* Meigen.

The larvae do not acquire their maximum sensitiveness until the end of the feeding period (Pouchet '72; Herms '11); as they approach the pupal stage they became sluggish, and if not actually less sensitive, are certainly very tedious to record. The most favorable age to make the light tests appeared to be at about the time of transition from the feeding to the migratory stage.

Like many other forms, the larvae soon become acclimated to light, and are much more sensitive if kept from one to three hours in the dark. But a long isolation from food and moisture tends to make them sluggish and to hasten the pupal stage. The best procedure is to keep the whole culture in the dark and not remove the larvae until immediately before they are to be used.

2. Variability of response

There proved to be a wide range of individual variability in the response to light, the behavior of a few of the larvae being so characteristic that they could be easily recognized by their reactions. For example, one larva had a preliminary exercise that it performed with great precision in each one of the experiments in which it was used. When placed with its anterior end away from the light, in the small wooden groove in which the larvae were started, this larva looped its anterior end directly back toward the light underneath its own body, until the 'head' emerged from under the posterior end and was struck by the full intensity of the orienting light; whereupon it promptly uncurled and crawled away from the light, responding otherwise like ordinary larvae. Out of several hundred larvae observed, I

have not seen this particular feat repeated. Though any 'extreme individuality' of this sort was uncommon, it was apparent that the rate of crawling and the response to light stimulation varied so greatly with different larvae under the same conditions that it had to be taken into account in a comparative study of a large number of individuals. To compare a larva of unknown sensitiveness, tested under one set of conditions, with an equally unknown larva tested under other conditions, would be of little value as an accurate basis for comparing the effect of the conditions. If one were attempting to find the precise effect of humidity on the time made by long distance runners, one would not try various runners of unknown speed, each under a different degree of humidity, and compare their 'times' as an index of the humidity effect. The only logical method would be to time the same runner under various conditions. His actual time would be an individual characteristic, but the relative time of the records under different conditions would be an index of the effect of humidity.

3. Standard test

But we cannot use this method on blowfly larvae, for the short sensitive period coupled with the rapid changes in the degree of sensitiveness with age, makes it impossible to complete a series of comparative experiments on a single larva. The nearest approach that can be made to such a method is to use, instead of a single larva, individuals which are as near alike as possible. For this purpose a standardization test, as it might be called, was devised and throughout the whole series of experiments only larvae testing to the uniform standard of sensitiveness were used.

During the experiments, each larva was kept in a separate, numbered box. The record of the standard test, as well as the subsequent trails, was made by putting a drop of very dilute methylen blue solution on the posterior end of the larva and letting it mark its own course on a sheet of paper placed on the observation stage. This method of recording the trails of larvae was used by Pouchet ('72) and Gross ('13). It has been repeatedly checked by control experiments with tap-water and appears to

change the reactions of the larvae in no way. The number of the box in which the larva was kept, in combination with the date of the experiment,³ made an identification number for all the trails of an individual larva. Thus the whole series of records was made permanent and can be referred to at any time.

The trails of the standard test were made in the following manner. A maggot was placed in front of the orienting light (fig. 1, *a*), which forced it to crawl toward the center of the stage in a line perpendicular to the ray direction of the mirror beams. When it was well on to the stage, the orienting light was turned out and at the same time the side light (fig. 1, *f**o*) thrown on, thus subjecting the larva to strong illumination from the left. The lateral illumination caused the larva to turn to its own right. After this trail had been completed, the side light was turned off, and the larva at once placed at the further end of the observation plate, where it was again started across the path of the mirror beams, this time under the influence of the opposite orienting light (fig. 1, *b*). The same side light (fig. 1, *f**o*) was then turned on, but as the larva was crawling in the opposite direction, the light now operated on the right photo-sensitive area, producing a turning again toward the observer's right, but toward the larva's left.

Figures 2 and 3 are photographs of actual trails made in this way, except that the methylen blue trail has been blackened with india ink to show better in the photographs. The sharpness of the bend in such a pair of trails is an index of the light sensitiveness of the larva, the symmetry of the curves, an index of the photo-sensitive balance. Only those larvae orienting to the new direction of light so accurately that both trails come to lie approximately parallel to the direction of the rays, were used in making the records compiled in tables 2 to 5. The amount of variation within the limits of accurate orientation, according to this criterion, is shown in trails *a*, *b* and *c* of figure 2, all of which meet the standard requirement of sensitiveness. Though there is a clearly marked response to the new direction of light

³ The method of writing dates employed in these records is the day, month, year, notation; thus 17/6/'13 is the 17th day of June, 1913.

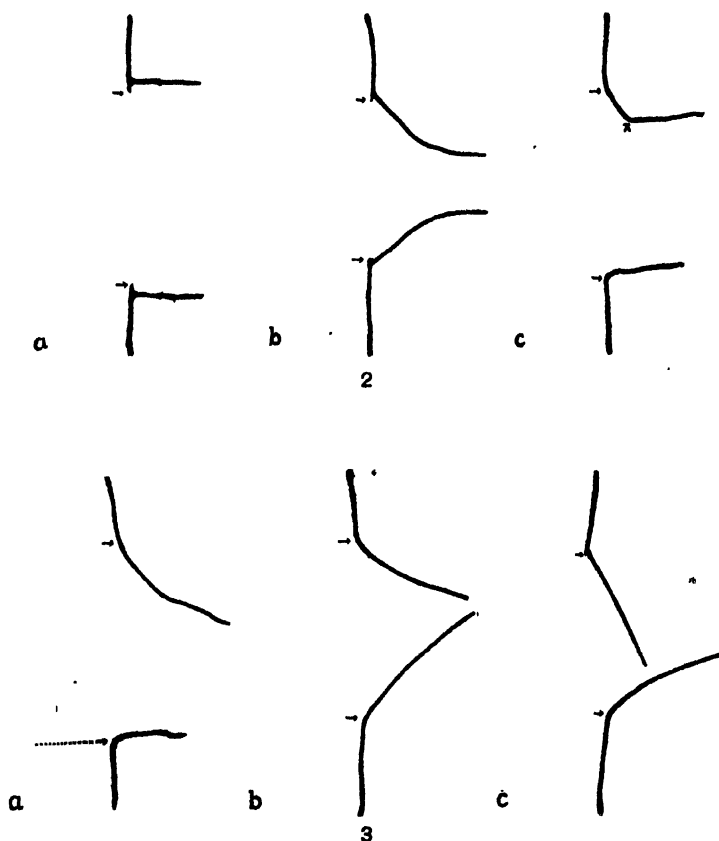


Fig. 2 Three examples of test trails which pass the standard orientation test. Each pair of trails was made by a different larva. Though there is some difference in the sharpness of orientation, each trail comes to lie parallel to the new direction of light.

Fig. 3 Three examples of test trails which do not pass the standard orientation test. Each pair of trails was made by a different larva. Though there is a response to the new direction of light in all cases, the larva fails in one or both of its trails to come to complete orientation.

in the trails shown in figure 3, *a*, *b* and *c*, these trails do not come to lie parallel to the new ray direction. These larvae were sensitive to the light but not sufficiently so to orient accurately and therefore were not used in making the records on which the tables are based.

In testing specimens from cultures at the optimum stage, from 15 to 25 per cent of the larvae were discarded because they failed to orient sharply to the change of direction in the light. Although the maggots from the same cultures were all of the same age, they were not necessarily in the same stage of development. The discarded larvae probably represented individuals that had passed, or not yet reached, their period of maximum responsiveness, rather than those which would never reach the normal degree of sensitiveness.

The larvae showing the desired accuracy of orientation were laid aside in numbered boxes and, after a rest, subjected to bilateral illumination from the mirror beams. Such a selection gave uniformity to the animals used in compiling the final results, and though it raised their standard of sensitiveness somewhat above that of the general population, it did not in any way distort the relative values of the subsequent records; on the contrary it is only by thus insuring uniformity in the material used that the comparison of results from different larvae under various experimental conditions becomes of value.

4. Plan of experiments

The plan of the experiments was to subject larvae of standard sensitiveness to lights of accurately determined intensity, with the conditions so arranged that the reactions could be measured in physical units. The rate of locomotion of the larva under the influence of light of various intensities has proved a difficult and unsatisfactory basis for a quantitative study (Mast '11, pp. 184-189). But orientation is a phase of the response to light which lends itself admirably to accurate measurement.

According to Loeb ('05, p. 2): "If two sources of light of equal intensity and distance act simultaneously upon a heliotropic

animal, the animal puts its median plane at right angles to the line connecting the two sources of light." It follows, as a corollary to this hypothesis that if the sources of light are unequal, a negatively heliotropic animal would deflect from the perpendicular toward the weaker light. This has already been demonstrated by Mast ('11, p. 179) for the blowfly larva.

By measuring in degrees the deflection toward the weaker light, physical units of measurement may be applied to the physiological phenomenon. And since with equal lights there is no deflection of the trail from the perpendicular to the line connecting the sources, whatever deflection there may be with unequal lights may be regarded as due to the effect of the difference between the lights. The starting point for a series of experiments of this sort would be at equality of the two beams of light. But it is not enough to know merely that there is no marked deflection when the lights are of equal intensity. It must be determined how many degrees from the perpendicular the aggregate response of a number of individuals falls. This necessitates not only a method of measuring each trail, but also a method of compiling the data obtained by measuring many trails made by different individuals.

The way in which we have obtained our results may be seen from a statement of (1) the method by which the trails were made, (2) the method of measuring single trails, (3) the method of tabulating the measurements.

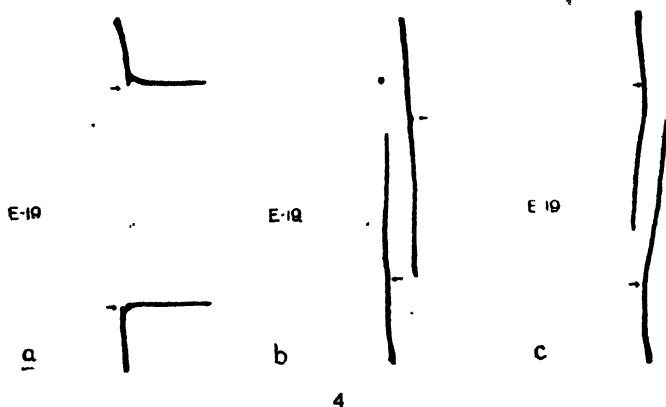
5. *Making the trails*

The handling of the larvae in making the records is of sufficient importance to be described in detail. At each end of the observation slate was a small wooden block, not shown in the diagram (fig. 1), with a groove in it leading down to the level of the slate. The blocks were located just in front of the orienting lights, the grooves extending along the line connecting the two lights (fig. 1, *a* and *b*). The larva to be tested was gently rolled from its box into the groove in front of the nearer orienting light (fig. 1, *a*), with its head away from the light. All other

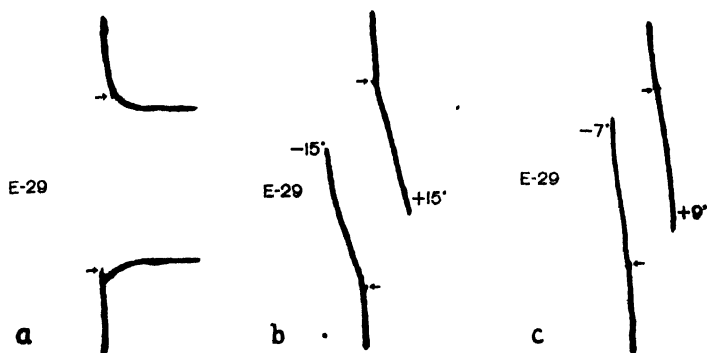
lights were shut off; the mirror beams by slides, and the opposite orienting light by a switch. The larva, started straight by the groove, was forced to move on to the paper in the direction of the orienting light, and so directly across the field of light from the mirrors. When it was well on the paper, the slides were pulled simultaneously from the two mirror beams, and at the same time the orienting light turned off. This subjected the larva to equal bilateral stimulation by the two beams of light, for the orienting light brought it into the field with its median plane at right angles to the line connecting the sources of light. Under these conditions there should be no deflection toward either light, and in fact there was none, for the larva continued in the direction in which it was started by the orienting light (fig. 4, *b*). At the end of this trail the maggot was allowed to crawl into its box, held edge down to the paper, then without being touched in any other way, it was rolled into the groove in front of the opposite orienting light (fig. 1, *b*) with its anterior end toward the center of the stage, and driven back across the field of light from the mirrors. The lateral beams were then thrown on the larva again and the second trail completed in the same way as the first. After a rest, a second pair of trails (fig. 4, *c*) was run in the same way, except that the order of running was reversed and the trail from the farther orienting light run first. These four trails, together with the test sheet, form the complete record of an individual and the unit for compiling the tables.

6. *Elimination of asymmetry*

But the trails do not all conform so closely to the theoretical response as those of figure 4. For example, those shown in figure 5 have a marked deflection under the same conditions of equal bilateral illumination. A careful study of this set will show the necessity of running the trails in pairs from opposite directions. In the lower trail (fig. 5, *b*), the larva deflected quite markedly to the left when the side lights were turned on, exactly as if the light from the right were stronger. But when its direction of crawling was reversed, it deflected to the right as if the left hand



4



5

Fig. 4 A set of trails made under equal opposed lights by a symmetrically responding larva; a, the test trails; b and c, trails under the influence of balanced lights.

Fig. 5 A set of trails made under equal opposed lights by an asymmetrically responding larva; a, the test trails; b and c, trails under the influence of balanced light.

light were stronger. The trails are nearly perfect mirror images of each other. Although this deflection is first to the observer's left and then to his right, it is in both cases toward the larva's left, for the direction of its crawling was reversed between trails. Such a response indicates an asymmetry in the neuro-muscular mechanism of the larva. The right side must be either more sensitive, or muscularly more active than the left for it gives a greater response to the same amount of stimulation. This asymmetry is by no means as uncommon as might be expected, in fact a certain degree of asymmetry is far more common than even an approximately perfect balance of sensitiveness. Whether the asymmetry is anatomical or physiological is a question which will be taken up later. The point we wish to bring out here is the method of eliminating the effect of such asymmetry, for it might easily be a source of serious error. Many markedly asymmetrical larvae were thrown out by the preliminary test (fig. 3, *a*). But many of those giving a perfectly symmetrical response in the test-trails showed marked asymmetry when run in the balanced lateral beams. The reason for asymmetry appearing in the later records, when it was not shown in the test trails, I believe to be this. In the case of the test trails, the larva is always orienting to a single light, and when orientation is attained, the anterior end of the larva is in the shadow of the posterior end. If the direction of light is changed, the larva keeps turning till it again crawls with its sensitive anterior end within its own shadow. If the 'head' swings out of the shadow, it is strongly stimulated and swings back again into orientation. Even if there were not a perfect balance of sensitiveness on the two sides, the lack of balance, unless it amounted to almost total insensitiveness of one side, would not appear under these conditions, for if, as the larva throws its anterior end from side to side in crawling, it passes beyond the boundary of the shadow, the change of intensity is sufficiently abrupt to produce a response on the less sensitive as well as on the more sensitive side, thus holding the animal within a course sharply limited by its own shadow.

When on the other hand the larva is made to crawl in a field of balance light, both lateral surfaces are illuminated, no matter

which way it turns, and the only means of maintaining its course is by a balance in the sensitiveness of the photo-receptive areas. This is clearly shown by trails such as those of figure 5. The guiding effect of the shadow is sufficient to obscure the unbalanced response in the trails of the test sheet and in the part of the trail made under the influence of the single orienting light. These trails lie almost exactly in the direction of the rays. But when the equal side lights are thrown on, which with a balanced larva should produce a direct continuation of the trail made under the influence of the orienting light, the asymmetry becomes at once evident in the deflection to the left. This larva, which gave a symmetrical test, proved to have the right side more sensitive than the left. Owing to some bilaterally unbalanced factor within the organism itself, it behaved, under the influence of equal lights, as if the right-hand light were stronger. Hence the deflection of a single trail is of little value in estimating the light effect. But turn the larva around and make it crawl into the field of light from the opposite direction (fig. 5, *b*), it still swings toward its own left side, but by reversing the direction of the crawling, the expression of asymmetry has been made to fall on opposite sides of the perpendicular to the line connecting the sources of light. If we measure deflection to the right in plus degrees and deflection to the left in minus degrees and add the two, the trails of an asymmetrical larva, run in pairs in opposite directions, are reduced to the theoretical response, and to the actual response given by perfectly symmetrical larvae.

7. Measurement and tabulation

The trails are measured as shown in the diagram (fig. 6). The lines xy and $x'y'$ are drawn through the trails at the point where the larva was when the side lights were turned on, and perpendicular to the line connecting the sources of light. A protractor is laid on the trail, with its center at the point where the lateral lights were turned on, and the deflection is measured in degrees. I have called this the angular deflection of the trail.

The measurements obtained from each individual were collected in tables such as table 2. The deflection of each trail is

entered in the appropriate column, and the sum of the total minus deflection and of the total plus deflection of the four trails is entered in the net column. The sum of the plus and minus deflections of a larva divided by the number of trails made gives

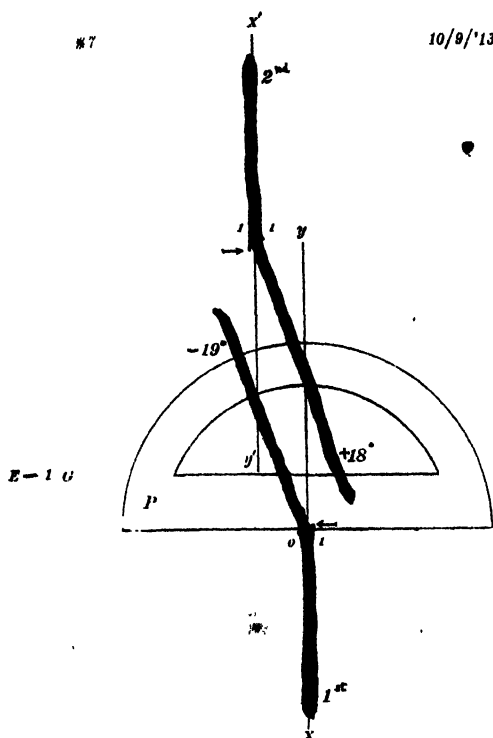


Fig. 6 Diagram to show the method of measuring trails. The lines xy and $x'y'$ are drawn through the trails at the points reached—marked by the arrows—when the side lights were turned on. The angle of deflection from this line is measured by a protractor, P . The small figures near the arrows indicate the number of wig-wag movements made when the side lights were turned on; 1st and 2nd refer to the sequence in which the trails were run; No. 7 is the box number, which, in combination with the date, gives the identification number of the larva.

the average angular deflection of the larva. The mean of the average deflections of a large number of individuals forms the final quantitative expression of the response to a given set of conditions. A study of this table shows that very few trails

TABLE 2
Equality. Four-glower intensity

NUMBERS OF THE LARVAE.	1ST SHEET		2ND SHEET		TOTAL —	TOTAL +	NET	AVERAGE ANGULAR DEFLEC- TION
	1st trail	2nd trail	3rd trail	4th trail				
No. 14, 13/5/'13	+10	-16	+15	-7	23	25	+2	+0.50°
No. 18, 13/5/'13	0	+6	+2	+5	0	13	+13	+3.25°
No. 20, 13/5/'13	+7	-8	+18	-12	20	25	+5	+1.25°
No. 15, 13/5/'13	+25	-11	-11	-3	22	28	+6	+1.50°
No. 4, 13/5/'13	+6	-6	+9	-7	13	15	+2	+0.50°
No. 22, 13/5/'13	0	+5	-7	-1	8	5	-3	-0.75°
No. 21, 13/5/'13	-5	-3	-21	+17	29	17	-12	-3.00°
No. 16, 13/5/'13	+17	-14	0	-6	20	17	-3	-0.75°
No. 11, 13/5/'13	-9	-1	+13	-6	16	13	-3	-0.75°
No. 9, 13/5/'13	-11	+5	-5	+3	16	8	-8	-2.00°
10 larvae					167°	166°	-1°	-0.025
40 trails								

conform exactly to the theoretical response. Out of the forty trails, only three show no deflection whatever. But the total plus and minus of each larva comes near to cancelling, and the average of the four trails of a larva is close to zero degrees deflection. The average response of the whole set corresponds almost exactly to the theoretical response, the average deflection from the perpendicular being only -0.025° . This is astonishingly accurate when one considers that a degree on the protractor of 7.6 cm. radius used in measuring the trails was only about 1.5 mm.

The striking results of table 2 were confirmed by similar results at various intensities of the equal beams, the details of which appear later in the paper. The immediate significance of these results is two-fold. The consistent closeness of the average trail to the perpendicular, and the equal distribution of the trails on either side of it, indicate that the method of individual measurement has effectively eliminated asymmetry and placed the different individuals on a uniform basis for comparison. The close conformity of the aggregate response to the theoretical response, when the lights are of equal intensity, makes a well grounded point of departure for a whole series of experiments with opposed

TABLE 3

Measurements of the trails of figures 7 and 8 in tabular form. No. 16, 22/2/'13, made the trails photographed in figure 7; No. 22, 30/1/'13, those of figure 8

NUMBERS OF THE LARVAE. DATE	1ST SHEET		2ND SHEET		TOTAL —	TOTAL +	NET	AVERAGE ANGULAR DEFLEC- TION
	1st trail	2nd trail	3rd trail	4th trail				
No. 16, 22/2/'13	-3	-12	-6	-11	32		-32	-8°
No. 22, 30/1/'03	-29	+17	-37	+8	66	25	-41	-10.25°
2 larvae					98°	25°	-73°	-9.125°
8 trails								

lights of unequal intensities. For if there is no deflection toward either side following equal bilateral stimulation, the deflection appearing under unequal bilateral stimulation may be regarded as a true expression of the physiological effect of the difference between the lights.

The same method of taking the records at equality was used with unequal lights. There is, however, one additional precaution to be observed. A given side of the larva should be subjected first to the stronger light in one pair of trails, and first to the weaker light in the second pair of trails, thus avoiding possible cumulative effects such as might result if the stronger light acted first on the more sensitive side in both pairs of trails. Asymmetry of response, though not cancelling to zero in this case, is checked out by the same method of running pairs of trails in opposite directions. The stronger light acting on the more sensitive side of the larva gives an abnormally great response; when the direction of the crawling is reversed, the same light acting on the weaker side gives a response correspondingly below the normal. The excess of one response is equivalent to the deficiency of the other, and the two average to a normal symmetrical response.

This is clearly shown by the two sets of trails photographed in figures 7 and 8, which were made under the same percentage difference in the lights, one by a symmetrical and the other by an asymmetrical larva. Tabulating these trails side by side, by the method already described, we obtain the results shown in table 3.

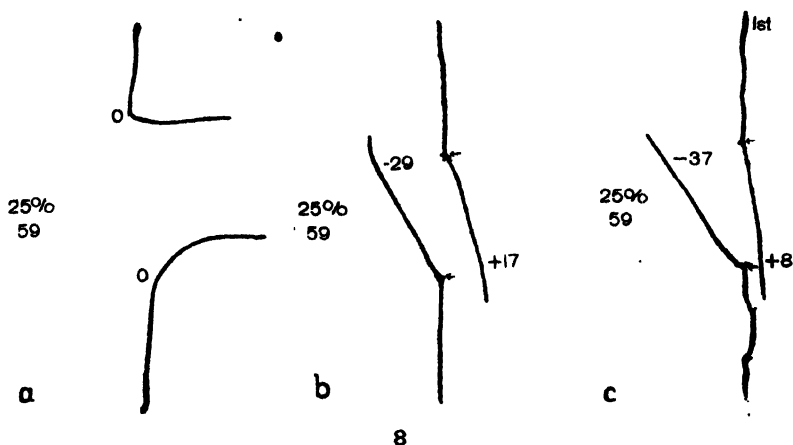
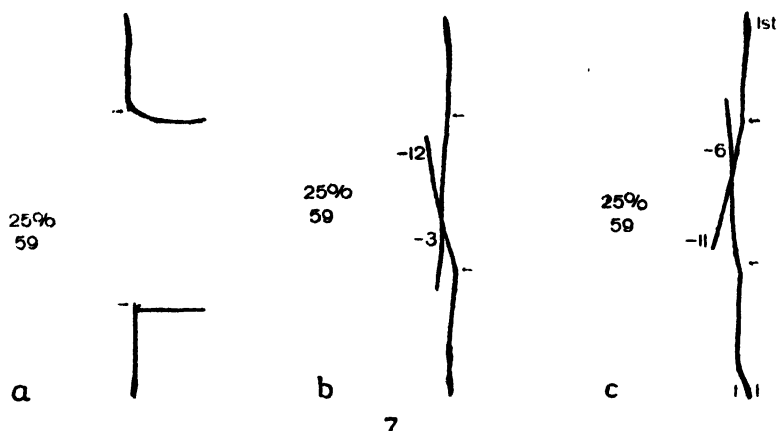


Fig. 7 A set of trails made under lights of 25 per cent difference, by a symmetrically responding larva; a, the test trails; b and c, the trails made under the influence of the opposed lights.

Fig. 8 A set of trails made under light of 25 per cent difference, by an asymmetrically responding larva; a, the test trails; b and c, trails made under the influence of the opposed light.

The total response of the four trails is nearly the same for the two larvae. Dividing the total response by the number of trails made, the average trail of the individual is obtained. This is for the symmetrical larva (No. 16, 30/2/'13), 8° , and for the asymmetrical larva (No. 22, 30/1/'13), 10.25° . A similar case at 50 per cent difference is illustrated in figures 19 and 20, page 260.

The conformity of these averages from such apparently different trails, together with the similar case already considered at equality, is convincing as to the completeness with which the disturbing effect of the asymmetry has been eliminated. I have laid special emphasis on this matter of asymmetry and the method of dealing with it, because it is important that the final results should be free from the possible cumulative effect of individual eccentricities. The average reaction of a large number of individuals, tabulated in this manner, is used to establish the angle of response under each set of conditions.

In spite of the precautions used in obtaining the records, extremely aberrant responses occasionally appeared. In all the tables, the average deviation of the individual responses from the mean of the set was computed, and when any records showed a departure of more than three times the 'average error,' they were not entered in the tables. This selection was not applied to separate trails but to the records of a larva as a whole. Either all the trails of a larva were accepted, or the larva was thrown out as an extreme variant and none of its trails were used. For these discarded trails, the trails of a fresh, tested larva, run under the same conditions, were substituted. The tables were computed both with and without this correction and the difference was found to be very slight, as the records exceeding three times the 'average error' were very uncommon, many of the tables not showing a single case.

TABULAR AND GRAPHIC STATEMENT OF RESULTS

Having perfected a system of measuring the reaction of the larvae to light, and established a fixed point of departure by means of the experiments with equal, opposed lights, we were able to attack the problem which actuated this work, namely, a tabulation of the reactions to opposing lights of different intensities, acting simultaneously on opposite sides of the animal.

It seemed advisable to begin at a well defined difference of intensity and work toward equality. Accordingly the apparatus was set up for a difference of 50 per cent between the opposing lights. By throwing glowers in or out of circuit, the absolute intensity of the common source of light could be varied at will, but however the source was varied, the relative intensity of the opposed beams remained the same. The reactions were all measured and tabulated as described in the preceding section, but as it would be impracticable to reproduce in detail over 30 tables comprising the measurements of 3000 trails it has been necessary to condense greatly the tabular statement of the results.

Table 4 was compiled from the reactions of larvae stimulated on opposite sides by lights of a constant difference of 50 per cent. The absolute intensities of the opposed beams (table 1) were varied by using from one to five 220-volt Nernst glowers as sources of light. Each column in the table shows the average response of 25 larvae, each run four times. The average angular deflection of these 100 trails is taken as the measure of the aggregate response to the special conditions. Table 4 is a condensation from five tables such as table 2, each column in table 4 representing the "Average angular deflection" column of table 2. In table 5 the statement of the measurements is still further condensed. By comparing tables 4 and 5 it will be seen that the 50 per cent column of table 5 corresponds to the "Averages for 100 trails" given at the bottom of table 4. Table 5, therefore, including as it does similar summaries of the measurements at all the other intensity differences used, is a tabular statement of the final results of the whole series of experiments.

TABLE 4
Angular deflection at 50 per cent difference

1 GLOWER	2 GLOWERS	3 GLOWERS	4 GLOWERS	5 GLOWERS
<i>Averages from four trails of a single larva</i>				
7.25°	19.25°	13.25°	20.25°	12.75°
19.00°	28.50°	19.25°	19.25°	15.50°
9.75°	21.75°	20.50°	24.25°	15.75°
25.50°	20.00°	34.00°	17.25°	29.25°
13.50°	13.50°	26.50°	19.50°	11.50°
24.25°	24.25°	22.50°	18.50°	16.75°
25.75°	32.00°	17.25°	19.00°	21.25°
26.25°	23.75°	18.50°	22.25°	19.50°
13.25°	23.25°	29.50°	22.50°	18.00°
21.75°	19.50°	16.00°	15.25°	21.00°
25.00°	13.25°	25.75°	11.75°	17.25°
27.75°	29.00°	39.50°	25.00°	24.00°
21.25°	21.25°	15.25°	17.00°	10.75°
18.75°	15.25°	22.25°	16.50°	23.00°
17.25°	17.25°	15.50°	28.50°	23.00°
23.75°	15.50°	11.50°	17.25°	9.00°
15.75°	23.00°	17.25°	8.75°	16.75°
21.75°	31.75°	27.25°	14.75°	14.50°
26.25°	27.25°	18.50°	16.00°	22.75°
23.75°	25.75°	17.00°	20.50°	21.00°
22.25°	20.00°	33.50°	17.75°	20.50°
10.00°	34.75°	18.50°	17.25°	22.75°
10.50°	24.50°	10.75°	20.25°	26.00°
9.25°	12.25°	14.00°	29.50°	24.75°
27.00°	20.00°	16.75°	27.25°	22.25°
<i>Averages for 100 trails</i>				
19.46°	22.28°	20.52°	19.88°	19.25°

TABLE 5

Based on the measurements of 3000 trails, showing the average angular deflection at five different absolute intensities and nine relative differences of intensity

	EQUAL- ITY	8½ PER CENT	16½ PER CENT	25 PER CENT	33½ PER CENT	50 PER CENT	66½ PER CENT	83½ PER CENT	100 PER CENT
1 Glower.....	-0.55°	-2.32°	-5.37°	-9.04°	-11.86°	-19.46°			
2 Glowera.....	-0.10°	-3.05°	-6.12°	-8.55°	-11.92°	-22.28°			
3 Glowera.....	+0.45°	-2.60°	-5.55°	-8.73°	-13.15°	-20.52°	-30.90°	-46.81°	-77.56°
4 Glowera.....	-0.625°	-2.98°	-6.60°	-9.66°	-11.76°	-19.98°			
5 Glowera.....	-0.325°	-2.925°	-5.125°	-8.30°	-10.92°	-19.25°			
Average.....	-0.09°	-2.77°	-5.75°	-8.86°	-11.92°	-20.23°	-30.90°	-46.81°	-77.56°

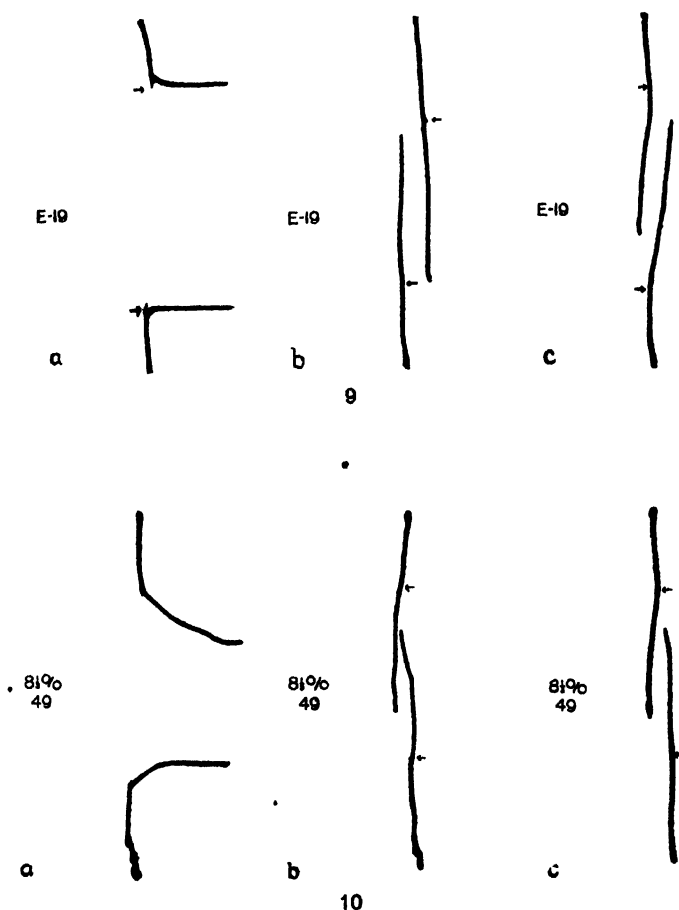


Fig. 9 The complete record of an individual larva run at equality, selected to represent the average deflection of all the trails made under the influence of equal lights.

Fig. 10 An average set at $8\frac{1}{2}$ per cent difference

Figures 9 to 14 show graphically the amount of deflection occurring at the various intensity differences. They are photographs of actual trails, selected because they represent the average deflection of all the trails run at the stated intensity difference.

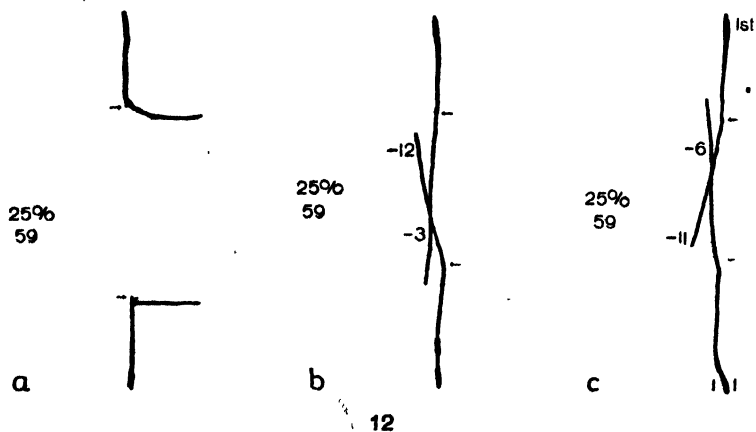
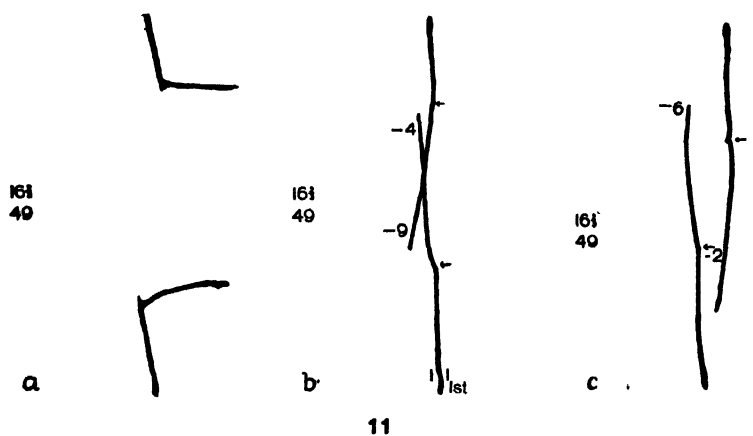


Fig. 11 An average set at 16 1/2 per cent difference

Fig. 12 An average set at 25 per cent difference

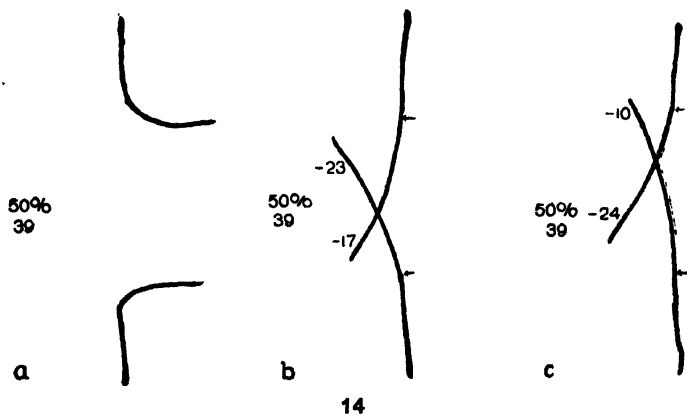
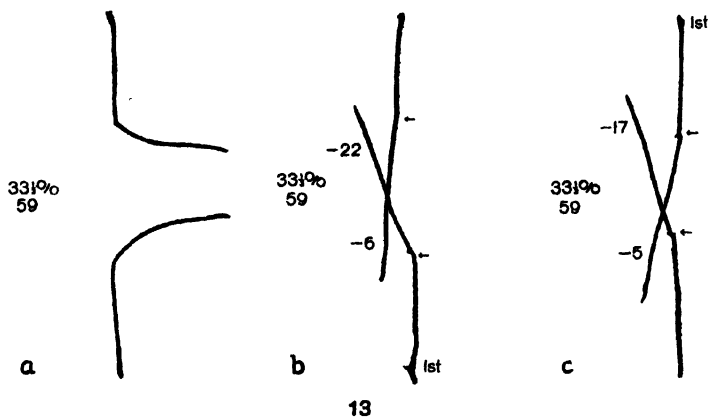


Fig. 13 An average set at $33\frac{1}{2}$ per cent difference
 Fig. 14 An average set at 50 per cent difference

In the subsequent theoretical discussion, the data have been somewhat rearranged according to the point under consideration. They afford (1) the possibility of constructing a curve of reactivity to differential bilateral stimulation; and (2) a means of arriving at several facts of interest in connection with theories of orientation, because the method of light control gave an opportunity to measure the simultaneous responses of opposite sides of the same animal to equal stimuli or to stimuli of known differences of intensity.

The constancy of the angular deflection at a given intensity difference (table 5) even though the absolute intensity of the lights was varied from one to five glowers, strongly suggests that the response follows the principle of the Weber-Fechner Law. A consideration of this phase of the reactions, however, has been reserved until further experiments can be made.

DISCUSSION

1. *The scale of reactivity*

a. Determination. From the data already presented a scale of reactivity covering the special conditions of these experiments may be determined for the blowfly larva. It has been shown that under equal opposed lights, there is no deflection toward either side in the aggregate response, and that if the intensities on opposite sides of the animal are made unequal, a deflection appears which becomes progressively greater as the inequality of the lights is increased. Apparently the relative difference of intensity determines the amount of the deflection, for it will be seen by referring to table 5 that the deflection remains practically constant for the five different absolute intensity determinations made at each of the percentage differences from equality to 50 per cent. The average of the five absolute intensity determinations at a fixed relative difference of intensity would therefore represent very accurately the aggregate response at the given ratio and the value of the average as determined for each percentage difference between the lights may be used to locate points on a curve which may be regarded as the blowfly

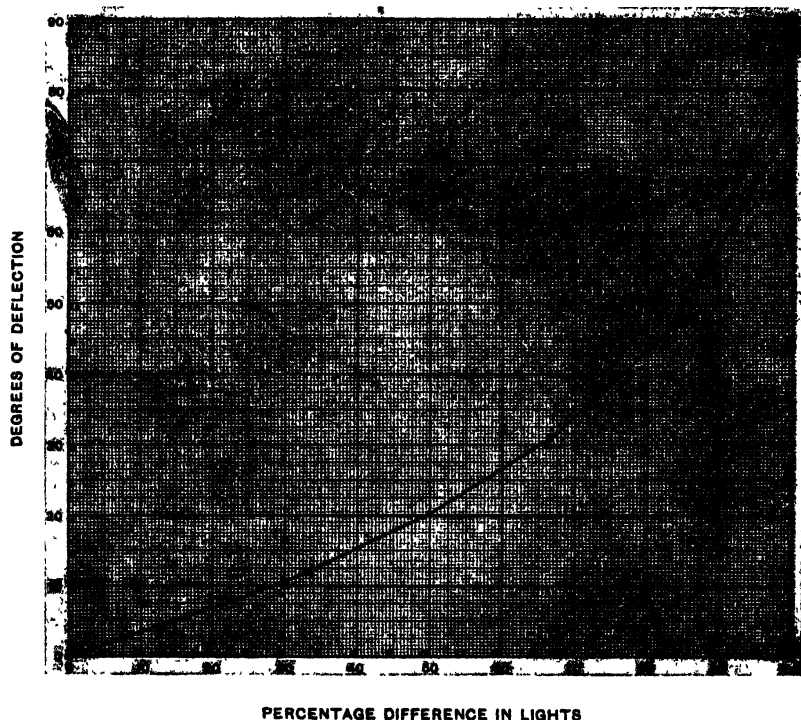


Fig. 15 A curve representing the angular deflection under opposed lights of graded differences of intensity, based on the measurement of 2600 trails; see also table 5.

larva's scale of reactivity to differential bilateral stimulation by light. Such a curve has been constructed in figure 15, plotting angular deflection along the axis of ordinates and percentage differences along the axis of abscissas. Figure 16 shows nine pairs of trails made at the nine intensity differences used to plot the curve of figure 15. These particular trails were selected because they coincided with the average deflection of all the trails run at the stated differences of intensity and therefore give a very clear picture of the experimental results on which the curve was based.

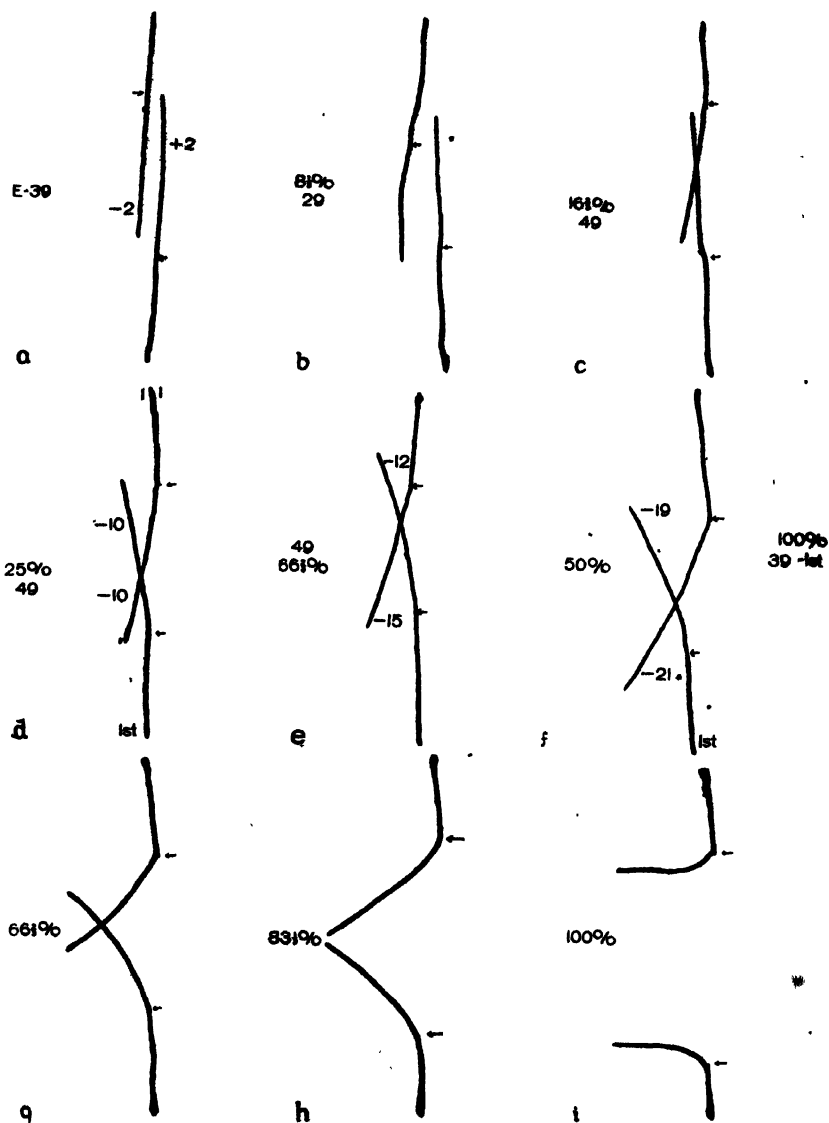


Fig. 16 A series of trails showing the progressive increase in angular deflection with increasing intensity differences between the opposing beams of light. Each pair of trails coincides with the average deflection of all the trails obtained at the stated differences of intensity; a, equality; b, $8\frac{1}{4}$ per cent difference; c, $16\frac{1}{2}$ per cent difference; d, 25 per cent difference; e, $33\frac{1}{3}$ per cent difference; f, 50 per cent difference; g, $66\frac{2}{3}$ per cent difference; h, $83\frac{1}{3}$ per cent difference; i, 100 per cent difference.

The consistency with which the deflection remained constant at various intensities of a fixed ratio made it seem unnecessary to run the whole series of intensities to establish the last three points on the curve, so the three-glower intensity alone was used at $66\frac{2}{3}$, $83\frac{1}{3}$ and 100 per cent difference. A difference of 100 per cent meant that one of the side beams should act against darkness, which was exactly the condition under which the preliminary trials were made. Accordingly, to establish this point, 200 of the test trails were measured. The deflection under these conditions should, theoretically, be 90° . But this is not the case experimentally, largely because of the time factor involved in orientation. The new orientation, even though it may be eventually a perfect alignment with the rays of light, is seldom instantaneously accomplished; and as locomotion is continued while the larva is orienting, the angle, if measured from the point where the light is turned on, is less than 90° . Figure 2 shows the varying angles in which the trails eventually come to orientation. But the time factor is of importance only when the relative difference is very great, and the amount of turning to be accomplished before orientation is attained is large. To ascertain its effect in the 100 per cent set, trails were measured in two ways: first, exactly as all the other trails were measured (fig. 6, p. 234); and again by measuring the final angle of orientation. Take, for example, the trails of figure 2, a, b, c; according to the latter method of measurement, all of them eventually attain a 90° -degree angle to the line of starting; but according to the former method, the time taken in orienting lessens the angle measured. The deflection at 100 per cent, measured as the smaller angles were measured was 77.57° ; the deflection of the same trails measured by their final orientation was 83.78° .⁴ The difference of 6.19° between the sets represents the time factor in a change of orientation through 90° . The results of the first

⁴ The measurements of the "final angle of orientation" were made at the point where the trails crossed the scale of the protractor of 7.6 cm. radius used in the other measurements. Had the measurements been made after the larva had been allowed to crawl through a longer distance, the determination for this set would have been nearer 90° .

method of measurement are shown in the curve, as a solid line, and those of the second as a dotted line (fig. 15).

b. Application. With a series of measurements of this sort established, not only the actual response at certain fixed intensity differences is known, but the consistent progression of the values of these determinations makes it possible to predict by reference to the curve plotted from them the response that would be evoked under any intermediate degree of stimulation. This should be of practical value as a comparative physiological measure of other experimental results.

For example, the responses of the blowfly larva to colored lights have been carefully worked out by Gross ('13, p. 475): "The colored lights . . . were obtained by cutting down the spectrum by means of diaphragms of blackened cardboard with narrow vertical slits of appropriate size." The lights used were therefore practically monochromatic, while the intensity of the beams was accurately measured by the radio-micrometer.⁵ Thus the two greatest errors commonly present in colored light work (i. e., the use of mixed light due to imperfect color screens, and the inaccurate determination of the intensity) were eliminated at the start.

Gross subjected the larvae to opposed beams of light of different wave lengths but of equal radiant energy, and recorded the deflection made toward one or the other of the lights. His figure summarizing the reactions under opposed green and blue lights of equal intensity is reproduced in figure 17. I have added to his figure the labelling of the sectors in degrees, and the pair of heavy lines which represent the average deflection. In making his records, larvae were started in the green light and allowed to crawl in the direction of its rays, along the 90-degree line of figure 17, until they reached the center of the circle. Here the blue light was turned on from the opposite side. The course then taken by the larva under the two opposed lights was recorded by indicating the sector through which it emerged from the circle. Figure 17 records in this way the points of emer-

⁵ For description of method by which the colored lights were equalized by the radiomicrometer, see Day, 1911.

gence of 46 larvae. Thirty-four emerged on the semi-circle toward the blue, and twelve on that toward the green. The trails above the horizontal (90°) diameter were made by turnings to the left, those below by turnings toward the right. If these trails had been measured in degrees of deflection from the vertical diameter,

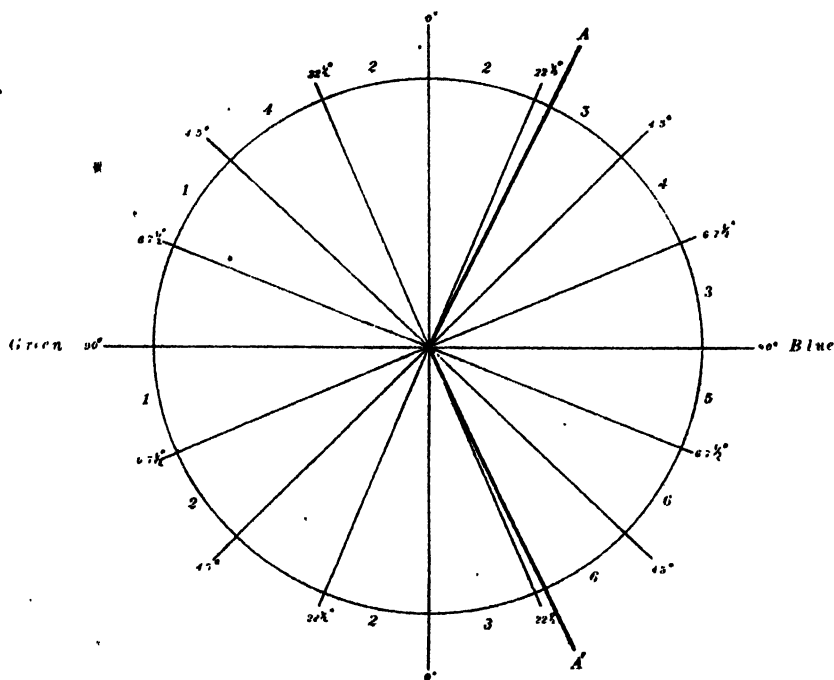


Fig. 17 A diagram to show the deflection of the blowfly larvae when subjected to opposed green and blue lights of equal intensities; after Gross, slightly modified. The numbers around the circle indicate the number of larvae that emerged through a sector. The heavy radii *A* and *A'* indicate the average angular deflection as approximately computed from the data presented by Gross.

the results would have been directly comparable to those presented in this paper for white lights. This deflection may, however, be approximately computed from the data of figure 17, by giving each trail the mean angular value of the sector in which it emerged from the circle. The trails to the left of the vertical diameter would be measured as minus, and those to the right as

plus. The sum of the plus and minus deflections, divided by the total number of trails, would give the average angular deflection toward the less effective color. The fact that certain of the larvae turned toward the color which in general acted more strongly, I believe to be due to the same asymmetry of responsiveness which, in my own experiments, caused the deflections toward the stronger of two white lights. The lack of balance of the larva in such a case would more than offset the difference in the colors, and the less effective color acting on the more sensitive side would produce a reflection toward the more effective color. In a large number of trails, however, this unstable asymmetry in sensitiveness would tend to balance out, and the average deflection would be a reasonably accurate expression of the relative stimulating powers of the two colors. In the case of green-opposed to blue, the average angular deflection was about 24° away from the green toward the blue. This angle of orientation could be attained by a turning either to the right or left and is represented by the heavy lines *A* and *A'* in the figure. As Gross has pointed out, this deflection is due to the qualitative difference between green and blue lights, for by actual physical measurement the intensities of the lights are the same.⁶ Comparing the angle of deflection under green and blue lights with that under unequal white lights, the angle of response corresponds to that produced when the difference in the intensity of the white lights is 56 per cent (fig. 15). In other words, green light is about twice as effective as a stimulus for the blowfly larva, as blue light. The values in the other color combinations could be estimated in the same way, but without access to the original records such computations would be only approximate and have therefore been omitted. There appears to be no other way in which the relative effectiveness of different pairs of colors can be estimated quantitatively. The method is presented more in the hope that it may prove of value in future work, than for the significance of the approximate relationship pointed out in this special case.

⁶ Gross also showed that with opposed lights of the same color and intensity, the average trail lay perpendicular to the line connecting the sources of light, thus establishing his zero point for the deflections obtained under different colors.

2. Theories of orientation

a. Relation of phototaxis and photokinesis. Among organisms that are visibly affected by light, there seem to be two distinct types of response. In some forms light appears to act as a stimulus to activity without any directive effect on position or locomotion. In other forms, light produces a definite orientation usually accompanied, in motile organisms, by locomotion toward or away from the source of light. For the first type of reaction, I have used the term 'photokinesis' (Engelmann '83); for the second, or directive response, 'phototaxis' (Davenport '97).

Holt and Lee ('01) maintained that the motor reactions of organisms to stimulation by light were not of two kinds, namely reactions to the intensity of light and reactions to the directions of its rays, but that light acted as a stimulus through its intensity alone, the direction of its rays serving merely to modify the intensity. With this conclusion I am in entire agreement. The terms 'phototaxis' and 'photokinesis,' as used in the following discussion, are applied *not* to reactions to ray direction and intensity respectively but to different types of reactions to the intensity of light. *

It appears from the work of Bancroft ('13) on *Euglena*, that light may operate as a stimulus either through its constant intensity, or through changes of intensity. In *Euglena* at least the mechanisms responding to constant intensity and to changes of intensity are distinct and separately modifiable. It should be clearly borne in mind throughout the following discussion that the term 'photokinesis' is not used synonymously with 'Unterschiedsempfindlichkeit' but to include any activity induced by light, *which does not result in orientation*, whether the activity be produced by constant intensity or by changes of intensity.

Probably the fundamental response to light is of the kinetic sort, that is, activity induced by light without the operation from within the organism of factors which screen or intensify its direct action, or of factors which indirectly distribute its effects. In such cases movement persists until fatigue ensues, or if there is a region of the environment where the stimulating agent is

of an intensity which does not produce a reaction, it persists until fortuitous movement takes the stimulated organisms thither with resulting aggregation in the so-called "region of optimum intensity."

It may be assumed that in this phenomenon the animal is so attuned to light that at some degree of illumination internal and external conditions are in equilibrium and 'stimulation' ceases. The intensity at which a particular form is unstimulated apparently depends on some internal factor bound up with the ultimate make-up of the organism. It may not be any more uniform than the amount of potassium or any other chemical present in the protoplasm of various animals.

In many organisms, the kinetic response to light is the only one present. The directed reaction, however, is never present without the kinetic response. It is not a primary phenomenon; it is the kinetic response with something added to it that comes from the association of the response with a specific organic structure, a structure that may localize the access of light and the material most sensitive to it, or that may localize the reaction. As a result, movements are restricted to locomotion in a more or less definite direction. It is not, therefore, in the fundamental reaction to light that the difference between photokinesis and phototaxis lies, but in the presence in the latter of factors which localize the action of the light or which limit the direction of the response.

b. Orientation in the blowfly larva. The orientation of the blowfly larva was taken up in the hope that a careful analysis of its typical directed response would yield some evidence as to the factors involved in phototaxis. Mast ('11) has devoted considerable space to the consideration of orientation in this form. His experiments are carefully carried out and accurately recorded. I have made free use of his results in this discussion.

The process of orientation under a single horizontal beam of light is the simplest aspect of the problem. But before this can be approached, the details of normal locomotion must be considered (Mast '11, p. 176):

The blowfly larvae move from place to place entirely by means of muscular contractions. They proceed somewhat as follows: the anterior end is raised, thrust forward toward one side, fastened to the substratum, and then the posterior end is pulled forward, after which the anterior end is again raised and thrust forward, now toward the opposite side, fastened, and the posterior end again drawn up. (I may add here that the posterior end is not merely pulled forward, but is also shoved ahead by the setting of the spines of the creeping pads.) The anterior end is thus turned alternately toward the right and left quite regularly during the process of locomotion. The extent of this lateral movement varies much, but it is usually great enough so that the extremity of the anterior end is nearly at right angles to the direction of locomotion.

If the larva is subjected to lateral illumination, as it crawls along in this manner, it may orient in one or two ways: it may stop crawling, raise the anterior end more than in the usual advancing motion, and wave it from side to side, finally fixing it away from the source of light and rapidly swinging the body into alignment; or it may proceed without any interruption of locomotion and without the conspicuous waving of the anterior end, simply swing a little more widely away from the light than towards it, as it extends the head first to one side and then to the other in the normal crawling movements. The most characteristic feature of the first method of orientation is the swaying of the raised anterior end from side to side. The term 'wig-wagging,' which Walter ('07) applied to a similar process in *Planaria* may be used to characterize this form of reaction. The swinging may be first toward the light and then away, or vice versa; it may be a single movement, followed immediately by locomotion in the direction in which the anterior end is fixed, or it may be repeated several times before locomotion is resumed. Whatever the details, and there are very many different individual modifications of the behavior, the result is a sharp turning of the anterior end away from the light, followed by the orientation of the rest of the body.

The function of the wigwag movement in orienting the larva is not altogether clear. Holmes believed that the direction in which the swinging first occurred bore no relation to the direction from which the stimulus came, but that the swinging was rather

one of a series of 'random movements' induced by the stimulus and served to 'try' the conditions on the different sides of the animal. He says ('05, p. 105): "If a strong light is thrown upon a larva from one side it may swing the head either toward or away from the light." Mast, repeating the experiment, recorded the direction in which the anterior end first turned, in a large number of trials. His table ('11, p. 189) shows a total of 177 turns away from the light and 165 towards it. At first sight this would lead one to conclude that the direction of turning is little influenced by the direction from which the light strikes the animal. But other experiments at different intensities did not confirm these observations, which like those of Holmes were made under intense lateral illumination. Mast found that when a larva was subjected to a moderate intensity of illumination, the turns away from the light were much in excess. This, he interprets in the following way ('11, p. 191):

When the larvae are first exposed to sudden lateral illumination in direct sunlight, they respond immediately by throwing the anterior end toward one side violently, no matter in what position this end chances to be. If it happens to be directed from the source of light when the sunlight is flashed upon the organism, it turns toward the source of light, and if the sunlight is immediately intercepted after the larva turns, it will continue in the direction toward which the anterior end points; if it is not intercepted, the anterior end is thrown in the opposite direction, and then the larva may follow this turn and become oriented immediately, or it may swing the end back and forth a few times before becoming oriented. If the anterior end faces the light when it is exposed to the sun, it is first thrown in the opposite direction and orientation takes place just as described above. The anterior end is thus turned in the direction opposite to that in which it is when the exposure is made. It is therefore evident that under these conditions the larvae will turn toward a strong unilateral illumination as often as from it.

Apparently this is not the case when the light is less intense (Mast '11, pp. 191, 192):

If the larvae are carefully observed when they are suddenly exposed to lateral illumination by diffuse light, it is found that they respond immediately only if the anterior end is turned toward the source of light when the exposure is made. If this end is in any other position, there is no reaction whatever until the organism, in its normal process of

locomotion, extends it toward the source of light. Then it is at once turned from the light to such an extent that it frequently makes a right angle with the posterior end. Later it is swung back but only part way. . . . But why does the organism turn toward the light, if the lateral illumination is very intense? Whenever the larva is stimulated, it turns the anterior end in a direction opposite to that in which this end is when it receives the stimulus. The tip of anterior end is relatively very sensitive; in diffuse light the larvae are stimulated only when this end is extended and fully exposed, but in very intense light, owing to the translucency of the surrounding tissue, it is stimulated no matter in what position the anterior end is; consequently if this end is turned from the source of light when the organism is exposed it is at once turned sharply in the opposite direction, i.e., toward the light.

This explanation of Mast's not only clears up an apparent inconsistency in experimental results, but also establishes a point the significance of which might be further emphasized. When the anterior end of the blowfly larva is stimulated by being turned toward the light, a contraction takes place on a *physiologically definite side*—the side on which the muscles are passively stretched.⁷ This is very suggestive of the motor reflex of a form like Stentor, where stimulation of the light sensitive region produces a "jerking toward a *structurally definite side*," the aboral pole. In one case the organism in its locomotion is put in touch with various regions of its environment by the swinging from side to side of a bilaterally sensitive anterior region, in the other case by a spiral turning which brings the single sensitive region first to one side and then to the other. In the blowfly larva, when the 'head' is swung to the left, it is partially shaded from a light coming from the right; when a Stentor rotates so its oral pole lies toward the left it also is partially shaded from the light coming from the right. The change of effective intensity when the sensitive region is turned from the shaded position toward the light produces a motor reflex which involves a change in the direction of locomotion. The reflex is repeated until a direction of locomotion is attained in which the changes

⁷ The tendency of a passively stretched muscle group to contract more readily than muscles already partially contracted is familiar in many reflexes, not only in cases where a nerve net is involved, but often where there are definite afferent and efferent nerves (see Uexküll, '09, chapter, Die Seeigel; Howell, '12, p. 21, etc.).

of intensity no longer occur. In both organisms, such a condition is brought about by an alignment of the axis of the body with the direction of the rays. In *Stentor*, when the alignment takes place, the spiral turning no longer produces changes in the amount of stimulus; when it is attained in the blowfly larva, the shadow of the posterior end of the body produces a shaded field in which the swingings of the anterior end do not involve any considerable changes in stimulation unless they are carried beyond the shadow, when the resulting motor reflex throws the head back into the shadow again.

Instead of orienting by the method just described, which is perhaps the most characteristic, the larva may swing into alignment with the rays of light by a different method. If a larva is suddenly subjected to a light from the side there is, in this second method of orientation, no cessation of locomotion and no wig-wagging. The anterior end simply swings a little more to the side away from the light than it swings in the normal locomotor movements, and the orientation is brought about gradually instead of immediately. Under the same conditions, one larva may orient by one method and the next larva by the other method. Moreover the same larva may use both methods of orientation. In figure 2, c, the upper trail was made by a combination of the two methods. When the light was turned on from the left, a gradual bending of the trail appeared, due to wider turnings away from the light than toward it. This was kept up to the point at which the trail turns suddenly (fig. 2, c, *). Here the larva stopped crawling, raised its anterior end, and performed several wigwag movements; finally it fixed its anterior end directly away from the light, and immediately came to a perfect orientation. What the cause of the sudden change of tactics was, I cannot say, but the sharpness of orientation at the place where the wigwag movements were made, in contrast to the gradual turning up to that point, is very apparent. The difference between these two methods of orientation, however, is not fundamental. The greater extent of the swingings termed 'wigwag movements' naturally brings about orientation more abruptly. In the case where the swinging is not so pronounced,

either because a lesser stimulus is acting or because a particular larva is less sensitive, it may become linked with the side-to-side movements of normal locomotion. Here the reflex contraction takes place just as before, but instead of being a separate, conspicuous wig-wag motion, it appears as a contraction added to that involved in the side-to-side swinging of the head in normal locomotion. This added contraction appears, of course, on the side away from the light and its effect is to produce a gradual turning away from the light until orientation is attained. In both cases when the anterior end is stimulated by a change of intensity, it swings in the opposite direction to that in which it was when the stimulus was received.

These two methods by which the blowfly larva orients to a single light are of such a nature that certain authors have called the process, orientation by 'trial and error.' Undoubtedly if we free the term from its original association with primitive intelligence, as they have attempted to do, it characterizes certain features of the orientation. But there are phenomena involved in the orientation of the blowfly larva to lights from two sources for which this interpretation is not adequate. The movements of a bilaterally illuminated head involve a distribution of the stimulus on the sensitive surfaces entirely different from that produced by the movements of the head when there is but a single source of light. A movement of the anterior end toward a single light produces a sudden increase in light intensity on the sensitive surfaces which is followed by a 'motor reflex,' throwing the head back into the shade of the body again. Motion toward the light is automatically prevented by the intervention of this reflex.

But with two lights of equal intensity acting on the larva from opposite sides, a swaying of the anterior end will encounter no changes of light intensity.⁸ Under these conditions there is,

⁸ The sources of light in these experiments were at such a distance from the observation stage that the change of 2 or 3 mm. from the central point, which the trial movements involved, would produce less than 0.3 per cent difference in the opposing lights. The minimum difference at which a definite change in response was detected was about 5 per cent.

therefore, no way in which the animal can 'select' from among the different intensities produced by its change of position, as the 'trial and error' interpretation demands.

Mast believes that the shading of the sensitive region by contraction, alternating with its exposure by extension provides changes of intensity which are effective in bringing about orientation. This explanation does not apply under conditions of bilateral illumination, for the change of effective intensity would be the same in whatever direction the anterior end was extended.

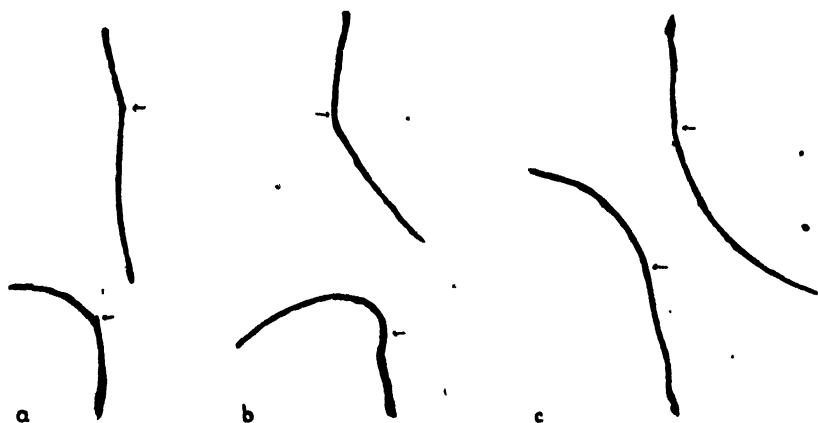


Fig. 18 Trails made by an asymmetrically sensitive larva under non-directive light; *a*, the test trails to horizontal lights, showing the asymmetry of response; *b* and *c*, trails of the same larva started in the orienting lights and subjected to vertical illuminating at the points marked by the arrows.

A definite orientation by the method of 'trial and error' where the 'trials' all result in the same amount of stimulation is hardly possible. Apparently the only explanation available is some form of the hypothesis advanced by Loeb, that equal intensity operating on symmetrically placed photosensitive areas produce a symmetrically distributed response. There is considerable experimental evidence that may be advanced in support of this view. Herms ('11, p. 207) blackened one side of the sensitive anterior region and found that when the animals so treated were subjected to non-directive light from above, typical circus movements were

produced with the blackened side toward the center of the circle. I tried a similar experiment with larvae that showed a markedly asymmetrical response to equal horizontal beams of light by subjecting them to light from above. The tendency to turn toward the less sensitive side was very marked. The turning, under non-directive light, of larvae that have been made artificially asymmetrical in their sensitiveness to light, and the similar turning of larvae naturally asymmetrical in their sensitiveness is hardly to be explained on any other basis than the assumption that unequal stimulation of opposite photosensitive areas is proportionately transmitted to the muscles concerned in locomotion. A significant fact in this connection is that the balance of reactivity in many of the larvae was found to fluctuate; a larva, which in one experiment gave a symmetrical response, often giving soon afterwards under the same experimental conditions, an asymmetrical one. Figure 20 (p. 260) is a photograph of the test trails, and six pairs of trails made by the same larvae under a constant intensity difference. The response is first 'left-handed,' *b*, (i.e., stronger to the left than to the right, implying that the right photosensitive area is more sensitive), then right handed, *c*, then symmetrical, *d* and *e*, then right-handed again, *f*, and finally symmetrical, *g*.

Walter ('07, p. 59) suggested that the asymmetry of response to light that he observed in planarians was due to internal irregularities. Undoubtedly asymmetry of response depends in many cases on anatomical differences in the opposite sides of an animal. In such cases the asymmetry probably does not change markedly in repeated reactions. But in cases like the one cited, where the stronger response is first on one side and then on the other, the balance which is disturbed must be a physiological one. We may assume that the processes of metabolism, or the previous reactions, or in short any of the factors which Jennings regards as effective in altering the 'physiological states,' have produced a change in the relative abundance or instability of the photosensitive chemicals of the receptive areas. Such an assumption is not unreasonable in view of the well known experiments of Loeb on the modifiability of light reactions by the use of chemicals; and the inducing by similar means of a light

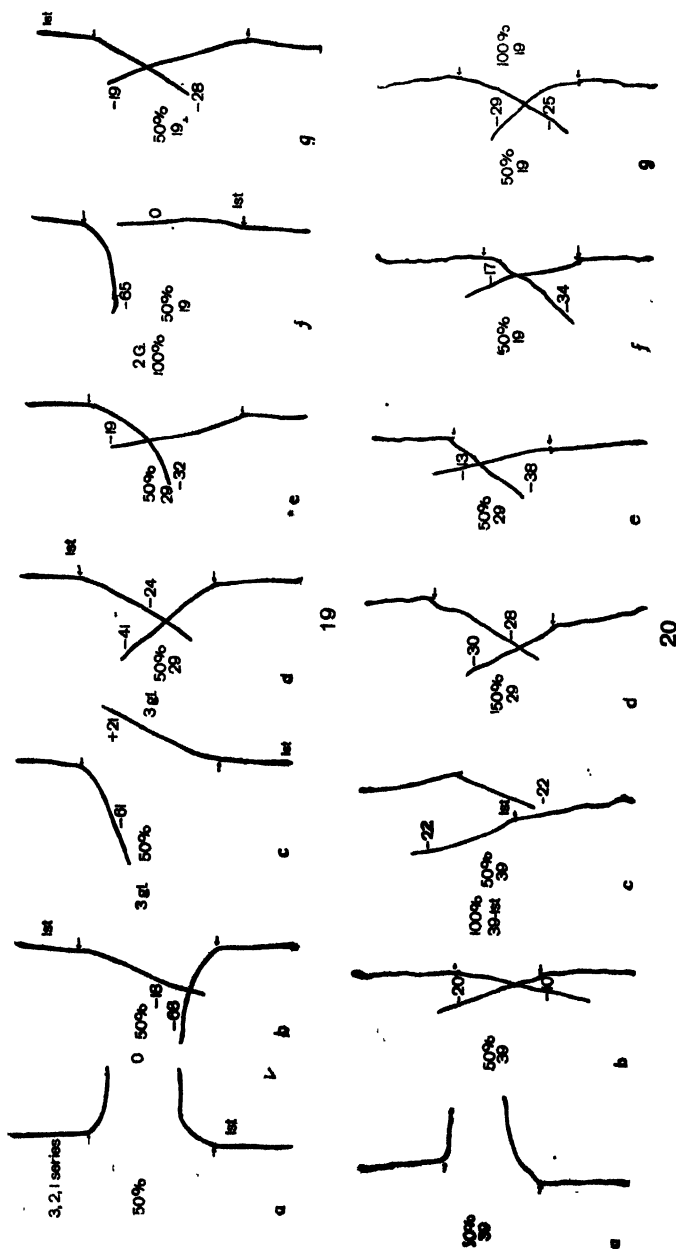


Fig. 19 Twelve trails made at 50 per cent difference by a larva which responded symmetrically throughout. Total deflection 288°; average angular deflection 24°; a, the test trails; b to f, trails made under the influence of the opposed lights. Fig. 20 Twelve trails made at 50 per cent difference by a larva which reacted with varying unbalance of response. Total deflection 307°; average angular deflection 25.5°; a, the test trails; b to f, trails made under the influence of the opposed lights.

NOTE.—The letters, a, b, etc., designating separate records are placed at the extreme left lower corner of the record.

response in an animal which was normally indifferent to light, as recorded by Moore ('13).

The cases of natural and artificial asymmetry of susceptibility to light are not the only indications that the light receptive system operates on the musculature bilaterally. The deflection of symmetrically responding larvae toward the weaker light is in every way comparable with the deflection of asymmetrical larvae toward their less sensitive side when the illumination of both sides is equal. In one case the unbalanced factor of the response is external and in the other case it is internal. Moreover, it is possible to balance the internal asymmetry of the photosensitive areas by subjecting them to a corresponding inequality of illumination from without. For example, suppose, when a larva is subjected to equal lights from opposite sides, it deflects 5° toward the left. By referring to the curve of figure 15, it will be seen that, with asymmetry balanced out, a deflection of 5° is produced by a difference of 15 per cent between the opposed lights. With the left-hand light 15 per cent stronger than the right, the same larva crawls straight down between the lights like a symmetrically responsive larva crawling under the influence of equal lights.

The physiological asymmetry has been corrected by a difference of 15 per cent in the bilateral stimulus. Thus we can not only demonstrate that certain individuals do not have a perfect bilateral balance of sensitiveness but we can measure the amount of the asymmetry and correct it by applying bilateral light stimuli of a corresponding difference in intensity.

The evidence that symmetrically located sensitive areas operate bilaterally on the musculature may be summarized as follows: (1) When the lights acting on the opposite sides of the larva are equal, the larva orients so that its median plane is at right angles to the line connecting the sources of light. (2) When the opposing lights are unequal, a deflection toward the weaker light appears. (3) Certain larvae are asymmetrical in their response, deflecting toward the less sensitive side when subjected to equal bilateral illumination. (4) The blackening of one side of the sensitive region produces a deflection toward

the side artificially made less sensitive. (5) Asymmetry of sensitiveness may be balanced by a corresponding inequality of the stimuli acting on opposite sides of the animal.

There seems to be no explanation for the response of the blowfly larva to opposed lights other than the assumption that symmetrical sensitive areas operate on the musculature of the two sides of the animal in proportion to the stimulation received. We do not know precisely what mechanism is concerned in the reaction, nor even what it is that is 'balanced' in the receptors. There are, however, certain general lines on which such a mechanism must be based. If the angle of orientation under opposed beams of light is such that the stimulation of the opposite sides is equalized, the receptive mechanism must be of such a nature that varying the axial position of the animal produces changes in the relative amount of stimulation received on opposite sensitive areas. Otherwise there would be no cause for the animal to assume a definite angle of orientation for each intensity difference between opposed beams of light. This equalization cannot be accomplished by a median sensitive area unless we assume that the area operates differentially on opposite sides of the median line, an assumption which throws us back again to bilaterality. Nor can it be accomplished by bilaterally located sensitive areas that are parallel to each other, nor by fixed eyes so placed that the tangents to the eyes at the optical axes are parallel. This can be made clear by a diagram such as figure 21. The heavy black lines represent light-sensitive areas of, let us say, 1 sq. cm. of surface. In figure 21, *a*, the sensitive surfaces, being perpendicular to the rays of light, intercept 1 sq. cm. of the light as represented by their projection on a plane at right angles to the rays of light (double lines of fig. 21, *a*). In figure 21, *b*, the planes have been rotated through an angle of about 45 degrees. This cuts down the amount of light falling on each sensitive surface. But since the surfaces are parallel, their projection on a plane at right angles to the rays of light will be the same, and the amount of light falling on the two sensitive surfaces will still be equal. The case illustrated by the diagram, figure 21, *c*, is essentially like that of figure 21, *b*. The retinas will receive light proportional to that which falls

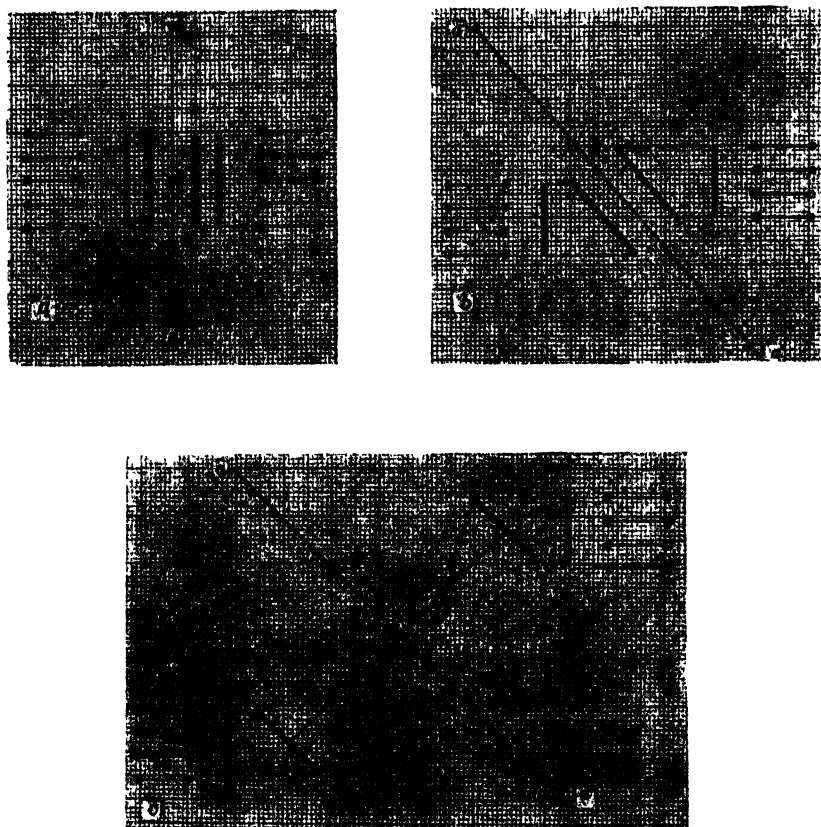


Fig. 21 A diagram showing that changes in the axial position of an organism with parallel sensory areas, or eyes with tangents at the optical axes parallel, do not change the relative amount of light intercepted by the sensory areas. In this figure, the lights, indicated by arrows, are assumed to be equal in intensity and opposite in direction.

on a plane paratangential to their optical axes at the focal distance of the dioptric apparatus. If these paratangents are parallel, rotation of the axes of the system will not change the relative amount of light intercepted by these planes. If, however, we assume that the sensitive surfaces are inclined at an angle to each other, the case is different. Figure 22 is a diagram constructed to show the conditions set up by rotation when the

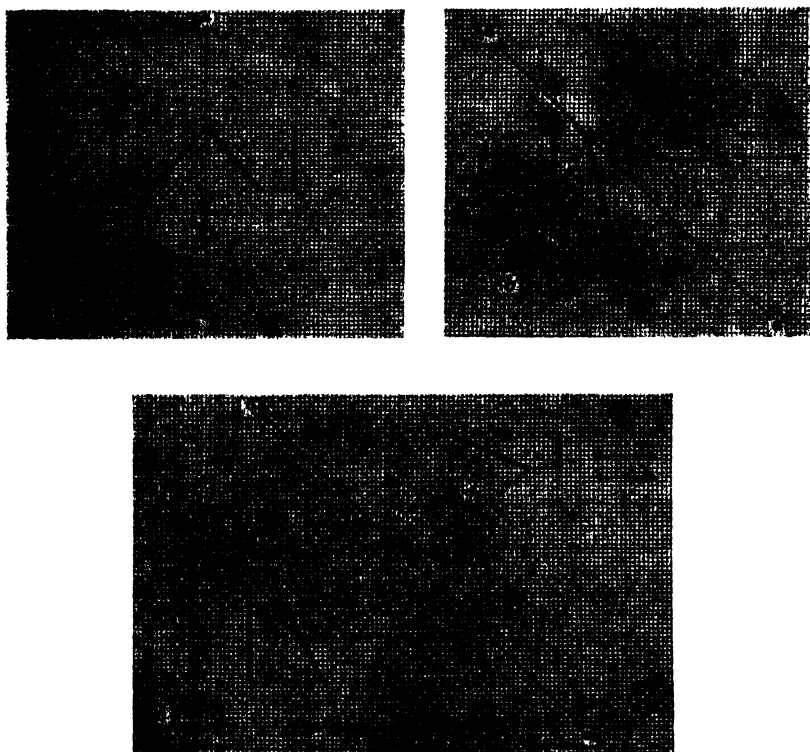


Fig. 22 A diagram showing that changes in the axial position of an organism with non-parallel sensory areas, or eyes with tangents at the optical axes non-parallel, change the relative amount of light intercepted by the sensory areas of opposite sides. Lights same as in figure 21.

sensitive surfaces are not parallel. In figure 22, *a*, the opposite surfaces intercept equal amounts of light because their angles of inclination are equal. In figure 22, *b*, the left area lies nearly at right angles to the rays of light and its projection is equal to its entire area, while the right side is inclined so that its projection is much less than its area. Consequently the amount of light acting on the bilateral photosensitive areas is different. In figure 22, *c*, the case is similar to that of 22, *b*. The amount of light received on the retina may not be exactly that operating on the paratangential plane at the focal distance of the eye, but it will be proportional to it.

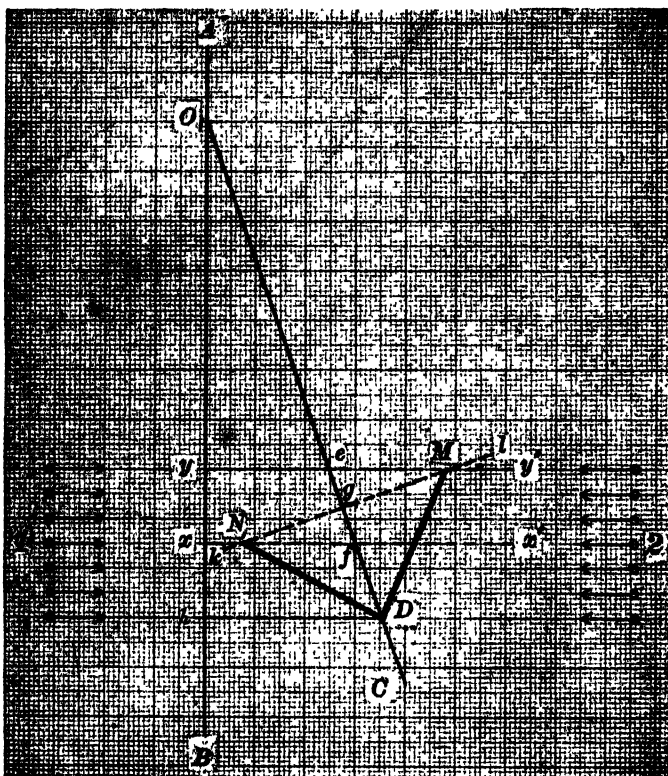


Fig. 23 Geometrical construction so show that when the sensitive surfaces are inclined to each other, a change in the axial position of the animal may equalize the light energy operating on them, although the intensity in the opposite sides of the field is unequal. The arrows indicate the direction, the numbers, the intensity of the lights; for construction and proof, see text.

We may assume, therefore, that the power to balance unequal bilateral stimulation, by a change in the position of the axis of the body depends on the inclination to each other of the photo-sensitive surfaces. The mechanism is simple and effective, and the explanation given fits the facts so far as they are known.

If this assumption be true, it should be possible to compute the angle of sensitive surfaces for an animal when we know its angle of orientation under varying inequalities of illumination. This angle has been computed for the blowfly larva, using the 'angular

deflections' already ascertained. The magnitude of the angle may bear no direct relation to the actual angle at which the sensitive areas are located in the body of the animal, because of the many factors which may modify the direction of the rays before they fall on the sensitive surfaces. The significant test of the hypothesis would be the constancy of the angle when computed from experimental data obtained under varying conditions.

The method of constructing such an angle is shown in figure 23, in which the opposing lights are assumed to be of a two-to-one ratio of intensity. The line AB is drawn perpendicular to the direction of the rays of light. On the line AB , construct angle BOC' equal to the actual average angular deflection of the larvae at a two-to-one ratio of lights (p. 245). The problem now resolves itself into the construction of an angle about OC as a bisector, which shall be of such a magnitude that equal distances on its opposite sides shall have projections on the line AB of the ratio of two to one.

Construction: From a point D on the line OC draw Dh perpendicular to AB . Lay off on AB distances hx and hy , such that $hy = 2hx$. From x and y erect lines perpendicular to AB , they will intersect OC at f and e respectively. Bisect the line ef , and at its middle point, g , construct a line kl perpendicular to OC . From the point of intersection of kl and yy' (M), draw a line to D . From the intersection of kl and xx' (N), draw a line to D .

The angle MDN is the desired angle

Proof: $eg = gf$ (construction)

Angle $egM = \text{angle } fgN$ (construction)

Angle $Meg = \text{angle } Nfg$ (alternate int. angles of parallel lines, yy' and xx' being parallel by construction)

Therefore triangle $Meg = \text{triangle } Nfg$ (side and two adjacent angles being equal)

$Ng = gM$ (similar sides of equal triangles)

$gD = gD$ (identical)

Therefore triangle $NgD = \text{triangle } Mgd$ (rt. triangles, altitude and base equal)

Therefore angle $gDM = \text{angle } gDN$ and side $DM = \text{side } DN$.

Now by construction hx is the projection of DN on AB and hy the projection of MD on AB , and by construction $hy = 2hx$

This fulfils all the conditions of construction

The equal lines MD and DN represent equal bilateral sensitive areas inclined to each other at such an angle, MDN , that the surface represented by MD intercepts an area of light twice as great as the surface represented by DN , its projection on the perpendicular to the light rays being twice as great ($hy = 2hx$). But the light falling on DN is of twice the intensity of the light falling on DM , so that the total amount of light received by each of the equal areas is the same.

By this method of construction, the average angle of sensitiveness was computed for four intensity differences, using as a basis the angular deflection of the larvae as determined by experiment. The angles are shown in figure 24. The magnitude of the angles is almost identical in all four cases. This angle, I would emphasize again, probably does not represent the actual position of the sensitive surfaces in the larva.⁹ There are too many modifying factors intervening between the direction of the rays of light in the field and the angle of incidence of the light on the sensitive areas to permit of basing any conclusions directly on the magnitude of the computed angle, for the structural peculiarities of the dioptric apparatus, or of the tissue overlying the sensitive area would change its value to a large extent. However, the computed angle would be constant in a given animal, and the angle of the sensitive surfaces would be equal to K times the computed angle where K may be defined as the 'structural constant' of the animal.

An interesting problem in connection with theories of orientation is whether light operates as "a constant directive stimulus" or whether changes in the intensity are the main cause of stimulation. Without entering into a detailed discussion, some experimental evidence bearing on this question may be presented.

⁹ The organs concerned with light reception in the blowfly larva have not as yet been identified. Pouchet ('72) concluded that the two pairs of cones on the maxillary segment were not the light sensitive organs, and suggests that the imaginal discs of the adult eyes may function in the larva. Certain unpublished experiments of my own confirm his exclusion of the anterior cones. I have not been able, however, to obtain any positive evidence as to the organs concerned.

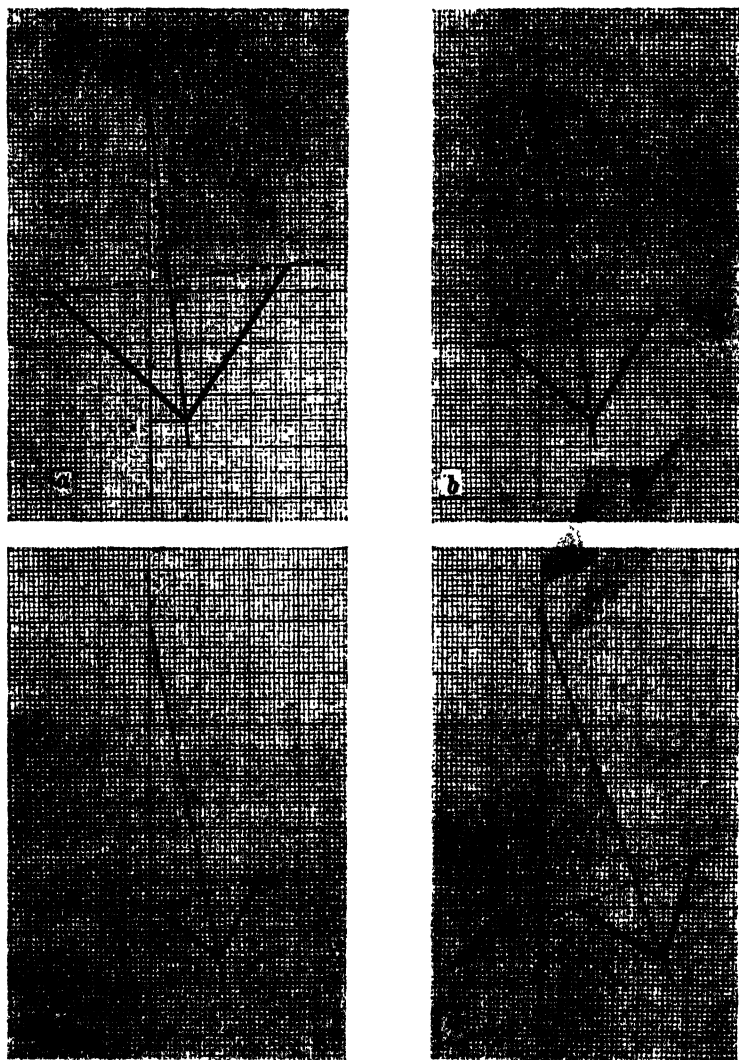


Fig. 24 Diagrams constructed after the method shown in figure 23; *a*, for 16½ per cent difference, constructed angle of sensitive surfaces = 82 degrees; *b*, for 25 per cent difference, constructed angle = 83 degrees; *c*, for 33½ per cent difference, constructed angle = 83 degrees; *d*, for 50 per cent difference, constructed angle = 82 degrees.

Recently Ewald ('13) has described experiments which prove that the Bunsen-Roscoe law holds for the response to light in the eye of *Daphnia*, as Loeb has maintained it should hold in animal light reactions (Ewald '13, p. 236):

This law states that in a light reaction the effect is proportional to the simple product of intensity and time. It was first proved to be true for the formation of hydrochloric acid from chlorine and hydrogen and for the blackening of silver chloride under the influence of light. Later it was found to apply to the phototropic curvature (Fröschel, Blaauw) of plants, as well as the human eye, though within rather narrow limits (Bloch, Charpentier).

If the law holds for the light reactions of photosensitive animals, intensity must of course operate as a constant stimulus.

Ewald found that the eye of *Daphnia* assumes "a definite normal position with regard to light" and that if while the animal remains fixed, the eye is subjected to lights from two sources, it takes up a definite axial position depending on the relative intensity of the lights. "In order to test the energy law, it is necessary to combine different light intensities with different times of exposure. If the product of time and intensity . . . is the same, the eye will always give the same reaction" (p. 236). This was proven experimentally by observing the position of the eye when subjected to one constant light and one light that could be varied at will. The variable light could be taken either from a constant low intensity source or through the apertures of a rotating sector wheel. An instantaneous shift from a slow, steady light to an intense intermittent light, delivering the same amount of light energy per second, caused no change in the position of the eye. If a sector wheel is used, giving too long or too short exposures to equalize the light, a change in the axial position of the eye appeared when the difference was greater than 10 per cent.¹⁰ "These observations prove that for the eye movements of *Daphnia* the energy law holds within the limits of accuracy characteristic of the reaction" (Ewald '13, p. 237).

¹⁰ In these experiments, the speed of the sector wheel was about 30 revolutions per second. With the reduction of the speed to 10 revolutions per second, a reaction was in some cases obtained when the change was made from steady to intermittent light.

Before the appearance of this paper, I had made a somewhat similar experiment on the blowfly larva, with the same results that Ewald obtained for *Daphnia*. Using the apparatus described on page 220, one of the beams of light was cut down by a diaphragm and the other by an episcotister, so that the light coming from one side was a steady beam of low intensity, and that from the opposite side an intermittent beam in which bright flashes alternated with darkness. The apertures in the sector wheel were adjusted so that the amount of light from each source was equal for a unit time. It has already been established that when the larvae are subjected to equal steady beams of light from opposite directions, the aggregate response is almost precisely at right angles to the line connecting the sources of light. The average angular deflection of 200 trails at equality (p. 240) was only 0.09° , when the degrees represented a distance of but 1.5 mm. If the Bunsen-Roscoe law holds for the phototactic response of the larvae, they should orient perpendicularly to the rays of light when subjected to the action of steady and intermittent lights of equal energy per second. The experimental results based on 136 trails made under these conditions show an average angular deflection of but 0.07° from the perpendicular.¹¹ These results seem to show that in the blowfly larva the phototactic reaction follows the Bunsen-Roscoe energy law.

Mast ('11, p. 234) says:

There is no conclusive evidence, except perhaps in animals with image forming eyes, showing that light acts continuously as a directive stimulus, that symmetrically located sides are continuously stimulated. . . . (p. 235). Light no doubt acts on organisms without a change of intensity much as constant temperature does, making them more or less active and inducing changes in the sense of orientation; but there is no conclusive evidence showing that light acting thus ever functions in the process of orientation.

Abrupt changes of intensity no doubt cause stimulation and are very effective in orientation under certain conditions¹² but they are not, as Mast maintains, the only ways in which light

¹¹ In these experiments the number of light impulses was 115 per second.

¹² See orientation of blowfly larva to single light, pp. 252 to 272.

operates to produce orientation, for our experiments on the blowfly, to test the Bunsen-Roscoe law, clearly show that light of a constant intensity both stimulates and plays an important part in orientation.

Holmes and McGraw ('13) in experiments on insects have come to the same conclusion in regard to the effect of constant intensity ('13, p. 373):

It is not possible we believe to construe phototaxis entire'y in terms of differential sensibility. Responses to the shock of transition, whether in the direction of an increase or a decrease of stimulation, may play a part in the orientation of many forms but the continuous stimulating influence of light appears to be in several cases at least the factor of major importance.

Bancroft's ('13) work, in which he showed not only that there was a distinct reaction to constant intensity present in *Euglena* but that it was largely the reaction to constant intensity which determined its orientation, shows the untenability of Mast's sweeping statement in one of the forms on which Mast himself worked.

The facts established concerning orientation in the larva of the blowfly may be summarized as follows:

1. When a larva is subjected to a single light, the changes of position, as it swings its head from side to side in the manner characteristic of locomotion and orientation, produce changes in the intensity of the light acting on the sensitive anterior end of the animal, due in a large measure to the shadow cast by its own body.

2. An abrupt change in the intensity of the light acting on the sensitive surfaces produces a reflex toward a "physiologically definite side"—the side on which the muscles are passively stretched.

3. Repetition of this reflex automatically checks motion toward the light.

4. In orientation to lights from two sources, the side to side swinging of the head does not produce changes in the effective intensity of the light on the anterior end as a whole.

5. Orientation to light from two sources depends on the relative amount of stimulation received by symmetrically located sensitive areas. This is shown by: perpendicular orientation to equal lights; deflection toward the weaker of two unequal lights; circus movements when one side of the sensitive areas is blackened; the presence of a natural asymmetry of response which may be counterbalanced by a corresponding inequality in bilateral stimulation.

6. An arrangement of bilateral sensitive areas may be postulated whereby stimulation on opposite sides of the animal may be equalized by a change of axial position. This arrangement accords with the facts so far as they are known.

7. The phototactic response of the blowfly larva depends to a large extent on the stimulating effect of constant intensity. The reaction to light of constant intensity follows the Bunsen-Roscoe law.

c. Analysis of factors involved in phototaxis. In the preliminary discussion of the relation of directive to non-directive light reactions, the interpretation was advanced that phototaxis had been evolved from photokinesis by the development, in connection with the latter, of certain factors which modify the action of the light on the organism, or indirectly distribute its effects. In the following section, an attempt has been made to analyze more closely certain of the factors involved in photokinesis and phototaxis, and to ascertain, as far as possible, their relative effectiveness.

The distribution of the stimulus in the field and on the sensitive surfaces of the animal, to a large extent determines the nature of the response. In a field uniformly illuminated from above there is, so far as we know, but one type of reaction, undirected activity which is maintained till muscular fatigue ensues or 'acclimatization' results. But if the field is not of uniform intensity and there are regions in it where stimulation does not take place, the organisms will sooner or later gather in those regions simply because they are not stimulated enough to move away from them. Under either mode of illumination, the reaction of the animal is of the photokinetic type; the condition which determines whether or not the aggregation takes place, lies not in the

reacting animal but in the distribution of the stimulus in the field. The term 'trial and error,' or better 'method of trial' may, I believe, be more appropriately applied to the method of aggregation by the kinetic reaction than to a method of orientation. The 'varied movements' of the locomotion of forms like *Stentor* or the blowfly larva take place whether or not the animals are stimulated by light. Though they undoubtedly play a part in orientation under some circumstances, it is by no means certain that they are essential to the attainment of orientation. In cases like that of a blowfly larva subjected to opposed beams of light, they probably play a very insignificant part in orienting the animal. It does not seem logical, therefore, to characterize the orientation of such forms as orientation by the method of trial, for even though there are some aspects of orientation that might be 'explained' on this basis, there are others which certainly cannot be so explained. An interpretation of orientation, to be acceptable, must accord not only with the details of a special case, but with all the varying details appearing under all the different manifestations of the phenomenon.

In the case of aggregation by the kinetic reaction, light causes the undirected locomotion which carries the animal into various environmental conditions. The movements initiated by light persist until 'selection' is accomplished with the cessation of stimulation, in other words, until the animal 'happens' to move into a non-photokinetic area. Such a reaction may be termed 'automatic distribution.'

A significant fact in connection with the operation of a uniformly distributed stimulus is that animals, such as the blowfly larva, which respond phototactically to horizontal light show a simple kinetic response when subjected to uniform illumination from above. The reason for this is the fact that under such conditions changes of axial position do not produce any difference in the relative amount of stimulation received by the sensitive areas. There is no basis for orientation. This establishes, as one of the critical factors in a directive response, a distribution of the stimulus such that a change in axial position on the part of the animal involves a change in the distribution or intensity

of the stimulus on the sensitive surfaces. For brevity, we may call a stimulus which fulfils these conditions a directive stimulus and one which does not, a non-directive stimulus.

Not all animals that are sensitive to light respond by orientation when they are subjected to the action of directive light. There must, therefore, be a second critical factor, a factor inherent in the responding organism. In the case of the blowfly larva, it was pointed out that orientation might be explained as a result of differential stimulation of bilateral sensitive surfaces inclined to each other at an angle, and the proportional transmission of that stimulation to the bilateral musculature. The explanation in this form will not apply to the case of an animal, like *Stentor*, which has but a single sensitive area. The two organisms, however, have in common a factor which I believe is a fundamental one in all directive reactions, a definite response to stimulation which is proportional to the intensity of the stimulus. In the blowfly larva there are bilateral sensitive areas, the stimulation of which produces a reaction bilaterally proportional to the amount of stimulation. In *Stentor* the spiral path of locomotion makes the single sensitive area perform the functions of two. There is a definite response to stimulation in the form of a swerving toward the aboral pole, and the amount of swerving is proportional to the stimulus. The operation of the response first on one side and then on the other brings about the orientation of the animal. Each animal has a method of reacting which is dependent on its structural peculiarities, but the animals respond in a definite and consistent way to stimulation by light, and both give a reaction proportional to the intensity of the stimulus.

When an animal does not respond to a directive light by orientation but merely by undirected activity, it is because the second critical factor of phototaxis is not present, the animal has no mechanism for producing a definitely directed response proportional to the intensity of the stimulus. We may assume that even in forms which do not respond directly, stimulation is proportional to the intensity of the stimulus, since the experimental evidence clearly indicates that stimulation, in its final

analysis is a chemical phenomenon and would follow the laws governing chemical reactions. The failure to respond directly is probably due, therefore, either to the presence of receptors of such a nature that changes of axial position do not affect the relative amount of stimulation received on opposite sides of the animal (fig. 21), or to the indefinite transmission of the stimulus from the end organs to the locomotor apparatus.

There are then two factors which must be present to produce phototaxis, one of these is resident in the stimulating agent, the other is resident in the organism. A directive stimulant may be ineffectual in producing orientation because of the absence in the reacting organism of a mechanism capable of directive response; similarly an animal with a perfectly developed directive mechanism may fail to orient because of the absence of directive distribution of the stimulating agent. The matter may be summarized thus:

Non-directive stimulant + indefinite response	= photokinesis
Non-directive stimulant + definite response	
proportional to the intensity of the stimulant	= photokinesis
Directive stimulant + indefinite response	= photokinesis
Directive stimulant + definite response pro-	
portional to stimulus	= phototaxis

The idea of a constantly acting stimulus which produces a reaction proportional to its intensity was one of the fundamental conceptions on which Loeb based his tropism theory. The fact that the theory as he advanced it postulated bilaterality in the responding organism has led to its abandonment by many authors who have studied the asymmetrically sensitive Protozoa. There is no doubt that in its original form the tropism theory does not apply to the orientation of asymmetrical organisms. The proportionality of the reaction to the intensity of the stimulant, within the range of normal physiological response, is however in my opinion the essential basis of any reaction involving definite orientation. Its method of expression varies in accordance with the structural peculiarities of the reacting animal, but the underlying phenomena are nevertheless in all cases essentially the same. If we include under phototaxis any reaction which involves a

definite axial orientation with reference to light, the tropism may be regarded as a special case of phototaxis in which the response depends on bilaterally placed sensitive areas.

The analysis of the factors involved in orientation as it has been presented here has been stripped of many details and limitations because I believe it has more value when stated broadly enough to allow details to be added as our knowledge of the subject increases.

I wish to express my indebtedness to Prof. G. H. Parker, under whose supervision this work was done, for his interest and helpful criticism, and to Dr. E. L. Mark for the facilities of the Zoölogical Laboratory and for the many courtesies shown me during my work there. I also wish to express the deepest gratitude to my father, who first aroused my interest in biology and whose criticism and encouragement have been a constant help.

SUMMARY AND CONCLUSIONS

1. The wide range of individual variability in the response of the blowfly larva to light renders a study based on untested animals of little value as a basis for comparing the effects of different experimental conditions.

2. To obtain reliable data for a comparative study of light reactions, it is necessary to establish a 'standardization test' and to make use of those individuals only, that show a uniform degree of sensitiveness.

3. To measure accurately the reaction to light in terms of definite physical units, the larvae have been subjected to the simultaneous action of opposed horizontal beams of light of known intensity, and the response measured, in degrees, on the resulting angular deflection of the trail.

4. When the opposing lights were of equal intensity, the average trail of the standardized larvae was within 0.09° of the perpendicular to the line connecting the sources of light.

5. When the opposing lights were unequal, the 'average trail' showed a deflection toward the weaker light. The amount of the

deflection was definite and constant, within the limits of experimental error, for a given intensity difference between the lights.

6. Using the average deflections obtained under each one of a graded series of intensity differences, a curve of the response to differential bilateral stimulations may be constructed. The deflection increases regularly with the increase of intensity differences.

7. The curve of response may be applied as a means of measurement to other experimental data obtained under similar conditions.

8. In orientation to a single horizontal light:

a. The changes of position due to the side to side swinging of the head characteristic of locomotion produce changes in the intensity of the light acting on the sensitive anterior end of the larva, due in a large measure to the shadow cast by the animal's own body.

b. An abrupt change in the intensity of the light acting on the sensitive surfaces produces a reflex toward a 'physiologically definite side,' the side on which the muscles are passively stretched.

c. Repetition of this reflex automatically checks motion toward the light.

9. In orientation to horizontal beams of light from two sources:

a. The side-to-side swinging of the head does not produce changes in the effective intensity of the light on the anterior end *as a whole*.

b. The attainment of orientation depends on the relative amount of stimulation received by symmetrically located sensory areas.

c. An arrangement of bilateral sensitive areas may be postulated whereby bilateral stimulation may be equalized by a change of axial position. This arrangement accords with the facts, so far as they are known.

10. The phototactic response of the blowfly larva depends, to a large extent, on the stimulating effect of constant light in-

tensity. The reaction to light of constant intensity follows the Bunsen-Roscoe Law.

11. The evolution of phototaxis is the result of the development, in connection with photokinesis, of certain factors which modify the action of light on the organism, or indirectly distribute its effects.

12. The critical factors of phototaxis are:

a. A distribution of the stimulant in the field such that a change in axial position on the part of the animal involves a change in the distribution, or intensity, of the stimulant acting on the animal or on its sensitive surfaces.

b. The presence, within the organism of a mechanism adapted to the reception of differential stimulation and a transmitting and motor apparatus that produces definite locomotor movements proportional to the intensity of the stimulation.

13. The 'response factor' may be present in the form of a bilateral mechanism, or in the form of a unilateral mechanism that reacts to both sides of the environment because of a rotational method of locomotion.

14. If we include under phototaxis any reaction which involves a definite axial orientation with reference to light, the tropism may be regarded as a special form of phototaxis, in which the response depends on the bilateral structure of the mechanism of response.

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THE BEHAVIOR OF THE EPIDERMIS OF AMPHIBIANS WHEN CULTIVATED OUTSIDE THE BODY

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SEVEN FIGURES (ONE PLATE)

In a previous paper¹ I have described the formation of strands and sheets ectodermic epithelium from pieces of amphibian larvae which were cultivated outside the body in lymph or plasma. It was shown that these extensions of epithelium were due to an amoeboid activity on the part of the cells at the edge of the extending mass of tissue. This year when opportunity for working on amphibian embryos and larvae was again presented, further observations and experiments on the behavior of the epithelial cells led to the discovery of several additional facts, as well as to the confirmation of the conclusions already drawn. Where not otherwise stated the observations here described were made on the larvae of *Diemictylus torosus*. The behavior of the epidermis from tadpoles of *Rana* and *Hyla* proved to be essentially the same as in the species mentioned. Studies were also made on the epithelial outgrowths from tissues of the adult frog.

Pieces of embryos were cultivated as before in lymph or plasma from the adult. It is very difficult to employ plasma on account of the rapid coagulation of the blood. Lymph has the practical drawback that in most amphibians a very small quantity is available while in the frog it varies so greatly that no uniform results could be obtained with it. Lymph tends to become too watery for use a short time after the frog is dead, and only a few drops of a usable quality can be obtained from any one animal. Epithelial cells do not wander out to any considerable

¹ Holmes, S. J. Behavior of ectodermic epithelium of tadpoles when cultivated in plasma. Univ. of Calif. Pubs. in Zool., vol. 11, 155, 1913.

extent unless they are in a medium of more or less firm consistency. When the lymph does not form a coagulation about the tissue no outgrowth occurs, although the cells may remain alive and the cilia upon them keep beating for weeks. In Ringer's solution epithelium from both embryos and adults will remain alive for a long time, but no outgrowth as a rule takes place. As so many cultures put up in frog's lymph proved unsuccessful the attempt was made to find a substitute for coagulum of blood and lymph which would elicit the same sort of thigmotactic reaction from the epithelial cells. After several experiments which proved to be failures, it was found that a mixture of Grüber's nutrient gelatin and blood serum afforded a culture medium that was very suitable for the purpose in hand. The proportions of the substances finally decided upon were about equal parts of serum and a two per cent solution of the gelatin. The gelatin solution was made and sterilized by boiling. In obtaining the serum, blood was drawn from the heart into a small glass tube and the clot removed. Then the gelatin warmed sufficiently to become fluid was mixed with an equal quantity of serum and centrifuged to remove the blood corpuscles, and the clear liquid was then transferred to another receptacle. The mixture thus formed becomes quite fluid when but slightly warmed and remains fluid for an hour or more at ordinary temperatures. One can work with it therefore without undue haste, and should the supply become solidified in the course of making preparations of the tissues it can be made fluid again by a slight degree of warmth. The same fluid may be preserved for several days if kept free from infection. I have found tissue to thrive in it after it had been prepared for several days as well as in mixtures freshly made. The ease with which this medium can be made and used greatly facilitates making preparation of tissues, and the results obtained by its use are also more uniform than those obtained by the use of plasma or lymph.

The ectoderm from pieces of young tadpoles grows out in this medium with great rapidity. In twenty-four hours the new growth may exceed the area of the introduced tissue and in

forty-eight hours it may be five or six times as great. Usually the ectoderm extends in the form of broad sheets, but sometimes narrow strands are seen which often branch, and occasionally some of the branches may meet and fuse with those of other strands. In many cases most of the epithelium had migrated away from the implanted piece, but the latter was in all cases covered by a thin layer of this tissue. It is common for outgrowths to appear both in contact with the cover slip and with the lower surface of the hanging drop, and they are about as frequent in the one situation as in the other (figs. 1 and 2). During the period of active extension the advancing epithelium is always furnished with an amoeboid border of very clear protoplasm as has been described by Harrison.² This border varies much in width and is often so exceedingly thin and transparent that it is very difficult to follow its course. The very fine processes that are sent forth are mostly in close contact with the substratum, and a number of observations make it evident that they possess considerable adhesiveness. The conclusion reached in my previous paper that the epithelial extensions are due to the amoeboid activity of their hyaline margin is confirmed by the discovery of several additional facts.

It was found that the epithelial membranes possess a remarkable degree of contractility. With the application of a stimulus a large epithelial extension may shrivel up to about one-tenth its original area. Often a very broad extension several times the area of the tissue from which it came may contract to a very narrow fringe, giving one the impression that to a large extent it went back to its original situation. The contraction starting at any one point may be seen to spread to surrounding areas until finally the whole mass may be involved. At other times only a small part of the sheet of ectoderm may draw back. In either case the margin of the retracted ectoderm becomes much thicker and rounded, the amoeboid processes disappearing. If the amoeboid margin is watched carefully in an epithelial extension that is just beginning to contract the

² Harrison, R. G. The outgrowth of nerve fiber as a mode of protoplasmic movement. *Jour. Exp. Zool.*, vol. 9, 787-848, 1910.

amoeboid processes may be seen to give way suddenly as if they could no longer resist the tension of the cells behind. The free margin then becomes thickened and rounded, and the cells take on a quite different form. When one part of the hyaline border gives way the adjacent parts follow; the retraction, however, may soon stop, or it may spread widely according to various circumstances. At the beginning of the contraction the pseudopods may be stretched out considerably, as the cells behind tend to draw away, before they become loosened from their attachment.

A characteristic of these epithelial outgrowths quite as striking as their extreme contractility is their extraordinary sensitiveness to slight stimulations. Bringing the slides from a cool place to the stage of the microscope where the temperature is a few degrees higher usually causes a retraction of some part of the margin of an epithelial outgrowth. The process of contraction was observed a great many times, and in fact it usually happens to a certain extent whenever slides are brought from a cool place for examination. It is probably the transition to a warmer environment rather than the higher temperature per se that causes the contraction, because preparations kept at room temperature form equally great extensions of epithelium, and in many cases the sheets of ectoderm which become contracted when exposed to a higher temperature subsequently extended again while the higher temperature remained unchanged. Contraction of any part of the sheet of ectoderm may be initiated by placing the point of a warm needle above the region in question.

That the cells are responsive to a mechanical stimulus was shown by touching the margin of the extension with a fine glass rod. Local contractions uniformly followed. Whenever a preparation was washed in Ringer's solution preparatory to furnishing it with a new supply of nutrient gelatine, the epithelial extension shrivelled up to a small fraction of its previous dimensions. By placing a drop of Ringer's solution upon the preparation the epithelium is caused to contract as soon as the solution diffuses into contact with it. Whether the contraction is due to a slight osmotic effect of the Ringer's solution, or to the stimulating influences of the salts is uncertain.

In several cases a part of the epithelial outgrowth of a piece of tissue was cut off and transferred to another drop of the culture medium. The act of cutting causes a violent contraction of the epithelial outgrowths, and the operation usually has to be done quickly to be successful. A broad and very thin sheet of cells may shrivel up, after it is cut off, into a small rounded mass that has very little resemblance to its previous condition. Subsequently these isolated pieces may spread out as widely as before.

The application of an unfavorable degree of heat causes a contraction of the epithelium quite aside from any stimulating influence of a change of temperature. Often the areas of epithelium may be broken up in this way into isolated masses of cells or even into individual cells. The influence of heat on epithelium is very similar therefore to its effect on the blastomeres of a dividing egg. The cells or cell masses tend to become rounded up and inert. Light has very little effect on the epithelial cells. If the heat rays are filtered out, epithelium may be exposed for hours to the most intense light without manifesting any evident reaction.

In general one may say that epithelial cells, like so many free organisms, respond to various unfavorable influences by contraction. When kept for several days in the same culture medium the epithelial cells show a tendency toward rounding up. This often results in the rupture of strands of cells, or the breaking up of sheets of cells into isolated masses. Under favorable conditions epithelial cells rarely isolate themselves from the general mass. It is probably the accumulation of products of excretion that causes the contraction of cell masses which are kept too long in the same medium. The breaking up occurs much more quickly in cultures kept at room temperature than in those kept in a cooler place, owing probably to the more rapid metabolism and the greater accumulation of toxic products. That it is not the injurious influence of room temperature alone that causes the contraction of the cells is shown by the fact that by washing the contracted masses in Ringer's solution and giving them a fresh supply of the culture medium, the epithelial cells may again form extensions at ordinary room temperature.

Cultures may be kept alive for a long time at room temperature, but they require more frequent washing and change of medium. Extensions of cells occur more rapidly at room temperature than when the cultures are kept in an ice box.

While it is clear that extensions of ectoderm are mainly due to the outwandering of cells, a certain amount of cell division is also found to occur. Division figures were not infrequently observed several days after the preparations were made. In two cases a division figure first seen in the prophase was watched continuously through the entire process of mitosis. The individual chromosomes could be distinctly seen and as many as fifteen could be readily counted, although the actual number is greater. The arrangement of the chromosomes in the equatorial plate, their pulling asunder, and the formation of the two daughter nuclei could be easily followed. The chromosomes appeared as V-shaped rods, and during the anaphase the open end of the V's were directed towards the middle of the division figure. Cleavage of the cytoplasm was completed soon after the telophase of the nuclear division. The whole process of mitosis was completed in less than three hours.

Mitoses become rarer the longer the tissue is kept in a given supply of culture medium. In one preparation from the larva of *Diemyctylus* mitotic figures were common four days after the tissue was isolated, and some were found on the seventh day, but none later. Changing the tissue to a fresh supply of culture medium, however, may cause cell division to be resumed. One preparation which had been subcultured several times showed numerous division figures fifty days after it had been removed from the body of the animal. Two days after its last transfer into fresh culture medium it showed over twenty-five mitotic figures. One of these was watched through into the telophase when the cell body could be distinctly seen to constrict into two separate cells. I have never seen mitotic figures so abundant as in this piece of epithelium which had been kept for fifty days in an artificial medium. The washing in Ringer's solution and its transfer to a fresh supply of fluid had apparently given the tissue a new lease of life.

As stated in my previous paper, extensions of epithelium often give strong evidence of amitotic nuclear division. Further observation not only furnished additional evidence of the same phenomenon, but revealed some of the conditions by which amitotic division is induced. Newly extended sheets of ectoderm present little or no indication of amitotic division. The nuclei of the cells are round or oval and rarely present any indentation of their outline. With epithelial outgrowths which have been kept for a week or more in the same culture medium indications of amitosis are more frequent. Instances of two, three or four nuclei in a cell are common (fig. 3), and in some cases as many as eight nuclei were seen in a single cell. No clear indications of cell division following the division of the nucleus were observed. In many cases the amount of nuclear material in relation to the cytoplasm was obviously increased to a very considerable extent. In all cases where numerous nuclei were seen in a cell there was a considerable amount of yolk present, although cells with very little yolk remaining in them frequently had two nuclei, but rarely more.

While evidence of amitotic nuclear division occurred abundantly in many preparations kept without a change of medium, other preparations made of the same material but changed every few days to a fresh medium showed no indication of amitosis, although they were kept much longer than the preparations in which the medium was unchanged. The appearance of indications of amitosis very frequently goes along with signs of diminished activity, such as the rounding up of cells, the disintegration of certain cells in the culture, and the general inactivity of the epithelial tissue. It is a commonly received doctrine that amitosis occurs most frequently in cells of declining vitality. Its association in cultures of epithelium with life under unfavorable conditions lends a certain support to this view.

Most of the epithelial outgrowths observed showed cells of somewhat different types. The ordinary cells of pavement epithelium (fig. C'), as soon as most of the yolk disappears, consist mainly of a clear, rather homogeneous protoplasm, more or less granular in the vicinity of the nucleus. A small amount of

yolk in the form of tiny spherules may persist until a late larval stage, even in the ectoderm of the tail and dorsal side of the body, while it is fairly abundant in the cells of the ventral side of the abdomen. As the cells become older and the yolk is gradually used up they tend to become relatively broad and thin, and to increase in transparency. Certain cells in early larval development become distinctly alveolar in structure (fig. 5). Such cells occur either singly or in small groups scattered about among the cells with homogeneous protoplasm. The alveoli are of various sizes in the same cell. These cells resemble and probably correspond to the Leydig's cells which have been described from the epidermis of several amphibians. I have observed the same type of cells scattered about in the epidermis of the young larvae of *Diemyctylus* in much the same way as they commonly appear in epithelial extensions in vitro. In the young larvae of *Triton*, *Salamandra* and *Siredon*, Maurer³ describes the Leydig's cells as scattered about singly among other cells of the deeper epidermis. On account of their vacuolated protoplasm these cells are considered as glandular in function, but they take no part in the formation of the cutaneous glands of later development. It is the appearance of the alveolar contents of the Leydig's cells that has given rise to the common opinion that they are mucous glands, but the cells have no direct connection with the exterior, and are usually covered with one or more layers of ordinary epithelium. In the preparations of epithelium in vitro various intergradations were found between ordinary epithelial cells and cells of the vacuolated type.

Occasionally also cells with granular contents occur in the epithelial outgrowths (fig. 4). The material forming the granules has a different appearance from the yolk, and probably represents the accumulated product of some sort of secretion.

As we have mentioned before, preparations may be kept alive for a considerable period if they are changed to a fresh culture medium. No especial attempt was made to find how long tissues could be maintained alive, but some of my preparations of epithelium were living over three months after implantation. One

³ Maurer, F. Die Epidermis und ihre Abkömmlinge.

cross section taken from the tail of a young *Diemyctylus* larva before it hatched from the jelly was put up in the middle of January. In a few days the epithelium showed extensive outgrowths along the cover slip and lower surface of the hanging drop. On January 27 after it had begun to show signs of deterioration it was washed and transferred to fresh gelatin and serum. Growth was resumed, and the cells showed a healthy appearance. The piece was transferred to fresh medium on February 2, and again on February 18. On February 24 it was divided into two parts. Both pieces, although their epithelial extensions became much shrivelled up after the transfer into fresh culture medium, soon put out extensive sheets of epithelium. Both pieces were transferred on March 3, when the same contraction and subsequent extension were again observed. One piece was transferred once more on March 10, and lived about a month longer. It is represented in figure 10. The second piece which showed signs of diminished vitality in the rounding up and separation of many cells and cell masses was re-transferred on April 1, after which it became active and sent out extensive sheets of epithelial cells.

The histories of many other preparations were essentially the same as the foregoing. In all cases the tissue was washed in Ringer's solution for a half hour or more, and the old gelatin mass around it removed before being put into a new supply of nutrient medium. A few preparations were left in Ringer's solution for a day without any apparent harmful results.

Epithelium remained epithelium throughout the experiments. The changes occurring in it were comparable to those taking place in the epithelium on the body of the larva. There was a progressive loss of yolk spherules, which in the epithelium from the dorsal side of the larva resulted in the total disappearance of yolk from most of the cells. As the yolk disappeared the cells became thinner and more expanded. From cells originally alike in appearance there came to be differentiation into cells of the vacuolated type, granular cells, although the latter were comparatively rare, ciliated cells, and cells of typical pavement epithelium. Whether the cells had received a start toward differ-

entiation into these different types before their implantation or whether their differentiation was entirely initiated after their transfer is uncertain.

A few experiments were tried with pieces of skin from the adult frog. Many of these pieces failed to show any epithelial outgrowth, although they were kept apparently in a healthy condition for weeks. In some instances large extensions of epidermis were observed. The cells at the margin of the outgrowths showed the same thin hyaline border with its minute changing pseudopods, as is found in the preparations of amphibian larvae. The general character of the epithelial outgrowth, the method of its formation and its behavior in relation to solid surfaces and to various stimuli are practically the same as in the extensions of larval epithelium.

Since my previously cited paper on the movements of epithelium in tadpoles was published, two papers have appeared which deal with the same subject. One of these is an article by Oppel⁴ which is devoted entirely to the epithelium of the tadpoles of *Rana*; the other, written by Osowski,⁵ includes a study of the movements of epithelial cells in tadpoles and in the embryos of fishes and of birds. The methods employed by both authors in their work on tadpoles are much the same. Tails of young tadpoles were cut off near the base, placed in diluted Ringer's solution for different periods, and then fixed and studied in serial sections so as to follow the way in which the cut surface came to be covered with epithelium. The covering of the cut surface was also followed by Osowski by making observations from time to time on the living tissue. No evidence of mitotic division was observed by Osowski in the epithelial cells that were extending over the cut surface. Both authors conclude that the movements of epithelial cells are responsible for covering the wound and that the movements in question are not the result of pressure or tension to which the cells may be subjected, but a con-

⁴ Oppel, A. Demonstration der Epithelbewegung im Explantat von Froschlärven. *Anat. Anz.* Bd. 45, 173-185, 1913.

⁵ Osowski, H. E. Ueber aktive Zellenbewegung im Explantat von Wirbelierembryonen. *Arch. f. Entw.-mech.*, Bd. 38, 547-583, 1914.

sequence of the activity of the cells themselves. Osowski, however, does not look upon the movements of the cells as amoeboid. The general conclusion to which he comes in regard to the movements of epithelium in the forms which he studied is:

Die Bewegung, die in den Explantaten zu Wundüberkleidung führte, gehört also zu der Rubrik der Selbstbewegung, und zwar zu den aktiven Massenbewegungen des Epithels, weil sie sich an einer zusammenhängenden Masse von Epithelzellen gemeinsam abspielt.

Vergegenwärtigen wir uns das im experimentellen Teil über den Vorgang der Wundbedeckung Gesagte und beachten wir die beigegeben Figuren, so wird man in der Bewegung alle Charakteristika der von Oppel so genannten "Epithelbewegung" wiederfinden: Sie vollzieht sich ohne Pseudopodienbildungen, die Zellen bewegen sich hinter- und nebeneinander sowie parallel zu der zu überkleidenden Oberfläche.

Osowski's failure to find pseudopodia is, I think, due to his study of preparations by means of serial sections, and to the fact that his studies of living material were made by observing the epithelium in contact with other parts of the implanted tissue. The pseudopods of epithelial cells of amphibian larvae are so short and so fine that it would scarcely be possible to detect them when they were extending upon other parts. Only when these epithelial cells become freed so that one can observe their free margin with high powers of the microscope is it possible to ascertain the real method of their movement. Apparently neither Osowski nor Oppel observed epithelium extending away from the implanted tissue. This was due doubtless to the employment of Ringer's solution, which does not evoke a thigmotatic reaction from the cells.

I am compelled therefore to dissent from the view that the movement of epithelium in amphibian larvae takes place according to the conception of "Massenbewegung" as described by Oppel⁶ and adopted by his co-worker Osowski. Study of epithelial cells under the favorable conditions of isolation where they are spread out on the under surface of a cover slip clearly shows the essentially amoeboid character of their movements.

⁶ Oppel, A. Causal-morphologische Zellenstudien. V Die aktive Epithelbewegung, ein Faktor beim Gestaltungs- und Erhaltungsgeschehen. Arch. f. Entw.-mech. Bd. 35, 371, 1912.

The behavior of epidermal epithelium *in vitro* serves to throw light on several features of the activities and functions of epidermis in its normal situation on the body. It is well known that abraded or cut surfaces are quickly covered by an epithelial layer. The chief factor in this process, as Oppel and Osowski have contended, is epithelial migration and not the growth or the mere passive movement of epithelial cells. Surfaces may be covered with little or no cell multiplication, but simply by the wandering of epithelium. The cells of the epidermis have an inordinate tendency to lateral spreading. They have a strong tendency to cling to their own kind of cells, and under normal conditions seldom become isolated from their neighbors. The adhesiveness of the newly formed hyaline pseudopods serves to give a point of attachment, and as the hyaline border advances the epidermal cells are pulled out more and more. Various observations have shown that the epithelial membranes formed were drawn out despite a certain contractility of their substance. The fact that as soon as the attachment of the pseudopods is broken the membrane draws back and begins to shrivel up indicates that the real seat of activity responsible for pulling out the sheets or strands of cells is the amoeboid movement of the advancing margin. The extending of this margin often causes the cells to become exceedingly thinned out. The membrane tends to creep out until the advancing margin comes into contact with other epithelial cells, thereby forming a continuous covering of the exposed surface.

The strong tendency of the epidermal cells to lateral spreading insures the formation of a continuous membrane; this is an important property in relation to several functions, and especially those related to osmosis. Studies made on the peritoneal cells of amphibians showed that the behavior of these cells has much in common with the behavior of the cells of the epidermis. There can be little doubt that the peculiarities of arrangement of the cells lining the body cavity, the endothelial cells of the blood vessels, and the cells lining various ducts and other passages depend upon certain features of cell behavior. It is quite feasible to study the characteristic features of the behavior of these cells by keeping them isolated *in vitro*. Considerable light

may be thrown on various morphogenetic processes in this way, but it is perhaps premature to attempt to speculate as to the value or promise of this method of analysis.

SUMMARY

The epidermis of amphibian larvae grows well in a mixture of equal parts of a two per cent gelatin solution and blood serum from the adult animal. Epidermis shows a marked thigmotaxis, and in hanging drop cultures extends away from the implanted tissue along the lower side of the cover slip and the lower surface of the drop of culture medium.

The extending mass of epithelium usually takes the form of a broad, thin sheet whose outer border is formed of very thin, clear protoplasm furnished with fine pseudopodia.

Sheets of extending epidermis are sensitive to slight stimulations, and show a remarkable degree of contractility, often drawing in to a small fraction of their previous superficial area.

Strong contractions of epidermis are evoked by thermal, chemical, osmotic or contact stimuli, but strong light has no appreciable influence on the epithelial cells. After contraction the epithelial mass may spread out as widely as before.

Epidermis from amphibian larvae may be kept alive for several months if it is transferred occasionally into fresh culture medium.

Mitotic cell divisions were observed in the living cells fifty days after implantation into the culture medium.

Amitotic nuclear divisions were found, especially in preparations that were kept for several weeks without a fresh supply of nutrient fluid.

Epidermal cells from embryos of *Diemyctylus* were seen to undergo differentiation in vitro into the different types of cells found in the epidermis of late larval stages.

Pieces of epidermis from the adult frog form extensions much like those from the larva, but growth is less rapid.

The extensions of the epidermis in both larval and adult forms are due to the amoeboid activity of the hyaline protoplasm along the margin of the extending mass.

PLATE 1

EXPLANATION OF FIGURES

1 Epithelial extensions from a piece of the tail of a *Diomyctylus* larva a week after implantation. The darker sheet of ectoderm represents the part extending in contact with the cover slip of the hanging drop culture; the lighter lower extension was in contact with the lower side of the drop. 1^a represents the original piece which was taken from a young larva before it had hatched from the jelly; it was quite opaque and showed no chromatophores which had become clearly differentiated a week later.

2 Epithelial extensions from a piece of tissue which had been cultivated for over two months, subdivided once, and subcultured several times. The dark central part represents the piece from which two large sheets of epidermis, an upper and a lower, have extended.

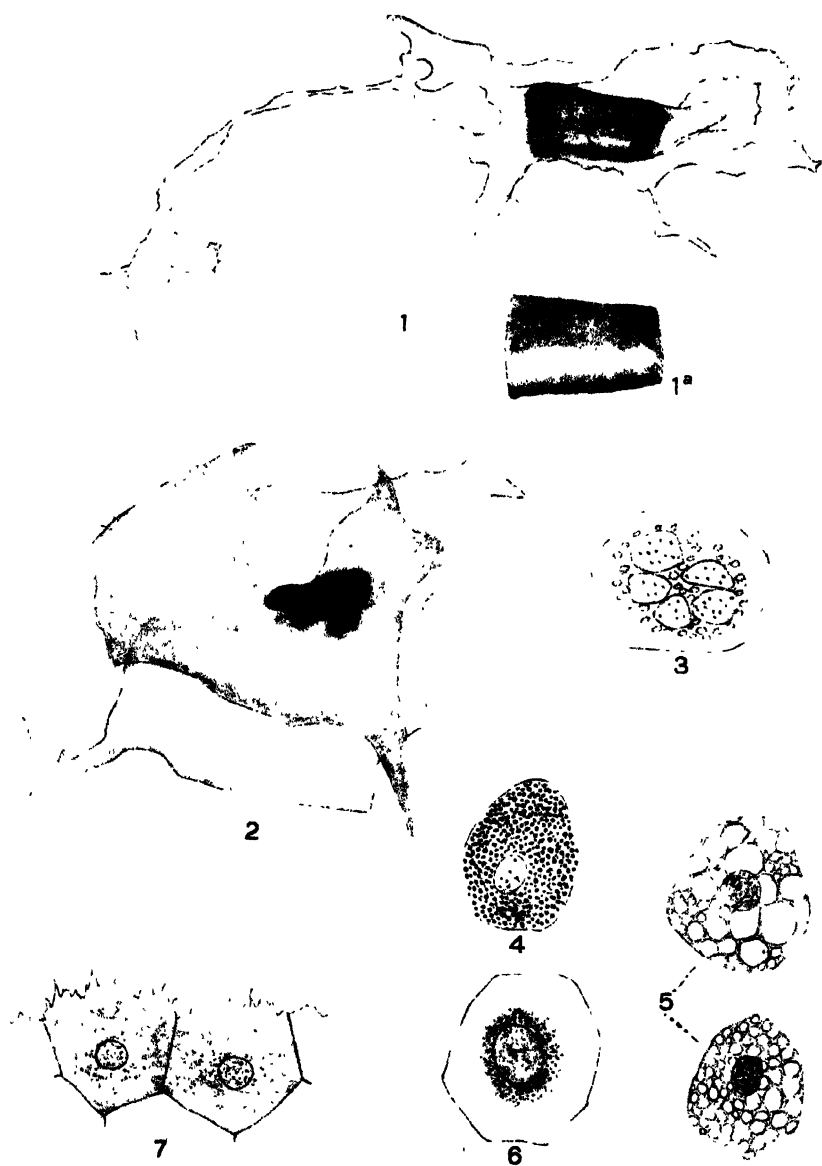
3 A multinucleated cell of epidermis.

4 Granular cell of the epidermis.

5 Two alveolar cells of the epidermis which differentiated *in vitro*.

6 Ordinary epidermal cell.

7 Epidermal cells from the edge of an advancing sheet of epidermis in contact with the cover slip. The thin hyaline border shows numerous fine pseudopods in contact with the glass.





EFFECT OF THYROID ON DIVISION RATE OF PARAMAECIUM

WALDO SHUMWAY

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THREE FIGURES

INTRODUCTION

The remarkable results recently described (Gudernatsch '12, '13) of a thyroid diet upon the development of the tadpole suggests interesting problems as to the effect of this substance upon the individual cell. In the following paper is given an account of the effects observed on the life history of *Paramecium aurelia* when particles of thyroid were added to its diet. The experiments here described were carried on in the Zoölogical Laboratory at Columbia University and in the Marine Biological Laboratory at Woods Hole, under the direction of Prof. Gary N. Calkins, to whom the writer's thanks are due for encouragement and advice.

In studying the effects of chemicals on *Paramecium*, many different indices have been employed by previous writers, especially chemotaxis, size, division-rate, and the phenomena exhibited by the gastric vacuoles. The rate of division has been chosen in these experiments as an index of the effects produced by the thyroid, as the investigations of Calkins, Woodruff and others have shown that it is very greatly affected by slight changes in the environment. Very recently Woodruff and Underhill have used this method in their series of investigations on "Protozoan Protoplasm as an Indicator of Pathological Changes" ('13-'14).

METHODS

All the experiments described in this paper were carried on upon a race of *Paramecium aurelia* descended from an individual isolated March 12, 1913 from a mass culture which had been maintained in the laboratory for several months. This race was hereafter maintained in four files¹ by daily isolations following the method described by Calkins ('02). The principal deviation was in the use of solid shallow watch glasses instead of depression slides. A daily record was kept of all the divisions which occurred in the history of the race, and these were averaged at the end of every five days to give the average division rate per day of the race for that period.

At the beginning of the experiments, two or three sister cells were isolated from each file, of which one was used to continue the control file while the others were used to start experimental files. In this way two or more lines of four files each were obtained of approximately the same protoplasm. Diagram 1 illustrates this method graphically. While one of the lines thus obtained was continued as the control line, the others were treated with thyroid or other substances as hereafter described. At the end of every five-day period, the division rates of the experimental lines were ascertained and compared with that of the control line for the same period.

The accompanying graph (diagram 2) shows the history of the control line (A) by five-day periods from the beginning of the experiment until December 20, 1913, when the line died out. The history of several of the experimental lines is indicated by dots and dashes.

The medium upon which the control line was kept was equivalent to one-half "standard hay infusion" (Jennings '10). It was prepared by boiling one gram of hay in one hundred cubic centimetres of tap water for ten minutes, filtering and allowing it to stand exposed to the air for twenty-four hours, when it was mixed

¹ The term "file" is used to designate a single genetic series of *Paramecia* derived from an original individual by the process of daily isolating one of the cells produced by the divisions of the preceding isolated cell.

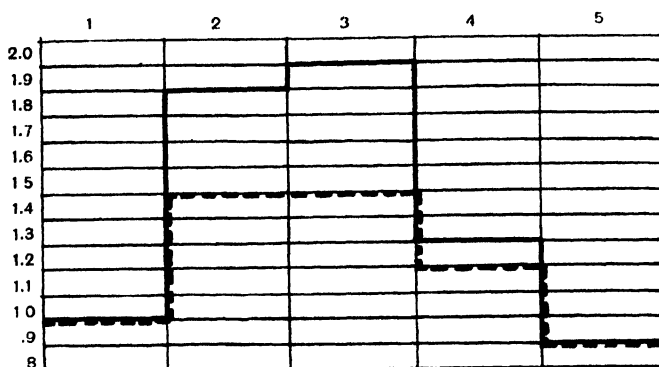


Diagram 1 To show ideal relations of sister lines of Paramaecia for comparing difference in division rate produced by changes in environment.

— = Control line - - - = Sub-line for experiment

with an equal quantity of boiled tap water. The quantity used for each individual was four drops.

The experimental lines were treated with the regular control medium to which a small quantity of thyroid or thymus was added. Whenever possible, glands from the same animal were used. At first emulsions from fresh glands were employed, later Armour's dried preparations. No attempt was made to render the emulsions absolutely bacteria-free. In the following accounts of the experiments, the emulsions used will be referred to by the numbers under which they are here described.

I. Thyroid Emulsion I and Thymus Emulsion I, were prepared from the glands of ten rabbits killed and dissected April 6, kept in physiological salt solution twenty-four hours, macerated and mixed one part to two parts boiled tap water. These emulsions were kept in a refrigerator at about 10°C. and were used during period 7, in the proportion two drops to two drops 1-2 Standard Hay Infusion.

II. Thyroid Emulsion II and Thymus Emulsion II were prepared from the glands of twenty rats killed and dissected April 13, kept in Ringer's Salt Solution twelve hours, macerated, ground, and mixed one part to two parts boiled tap water. These emulsions were kept in the refrigerator, and used during period 8 in the proportion two drops to two drops 1-2 S.H.I., and during periods 9 to 12, in the proportion 1-4 drop to four drops 1-2 S.H.I.

III. Thyroid Emulsion III and Thymus Emulsion III were prepared from the glands of nine rabbits killed and dissected April 25, kept in physiological salt solution five hours, macerated, ground, and mixed one part to two parts boiled tap water. These emulsions were kept in the refrigerator, and used in Experiment 1, periods 13 to 15, and in Experiment 2, in the proportion 1-4 drop to four drops 1-2 S.H.I.

IV. Thyroid Emulsion IV and Thymus Emulsion IV were prepared from cow thyroids furnished me by the kindness of Dr. Gudernatsch. On June 28 these were macerated, ground and mixed one part to two parts boiled tap water and used in Experiments 3 and 4, during periods 23 and 24, in the proportion 1-4 drop to four drops 1-2 S.H.I.

V. This symbol will be used for preparations made daily from Armour's Thyroids U.S.P. (desiccated) and Armour's Thymus (desiccated) by shaking up 1.5 mg. in 2 cc. boiled tap water and adding 2 cc. 1-2 S.H.I. Four drops of these mixtures were used at each isolation.

EXPERIMENTS

Experiment I. April 9 to May 23, 1913.

Average Division Rate

PERIOD	A (CONTROL)	D (THYROID)	E (THYMUS)	F (THYROID-CONTROL)	LINE
7	1 50	2 70	0 55		
8	1.05	1.95	1.10		
9	1.00	2 90	1.10		
10	1 55	3 60		1 90	
11	1.45	2.80		1.40	
12	2.20	3 15		2.25	
13	0.95	2 15			
14	1.75	2.60			
15	1.00	2 40			

For this experiment two lines of four files each were derived from the control line (A), one of these (D) was kept in 1-2 S. H. I. to which a small quantity of thyroid (Thyroid I, II, III, see above) was added, the other (E), not shown on diagram 2, was

Diagram 2 Chart showing life history of line A, together with the differences produced in sub-lines treated with thyroid, some of which were thereafter returned to control conditions.

----- = Control line

. = Lines treated with thyroid

- - - - - = Lines returned to control conditions after thyroid treatment

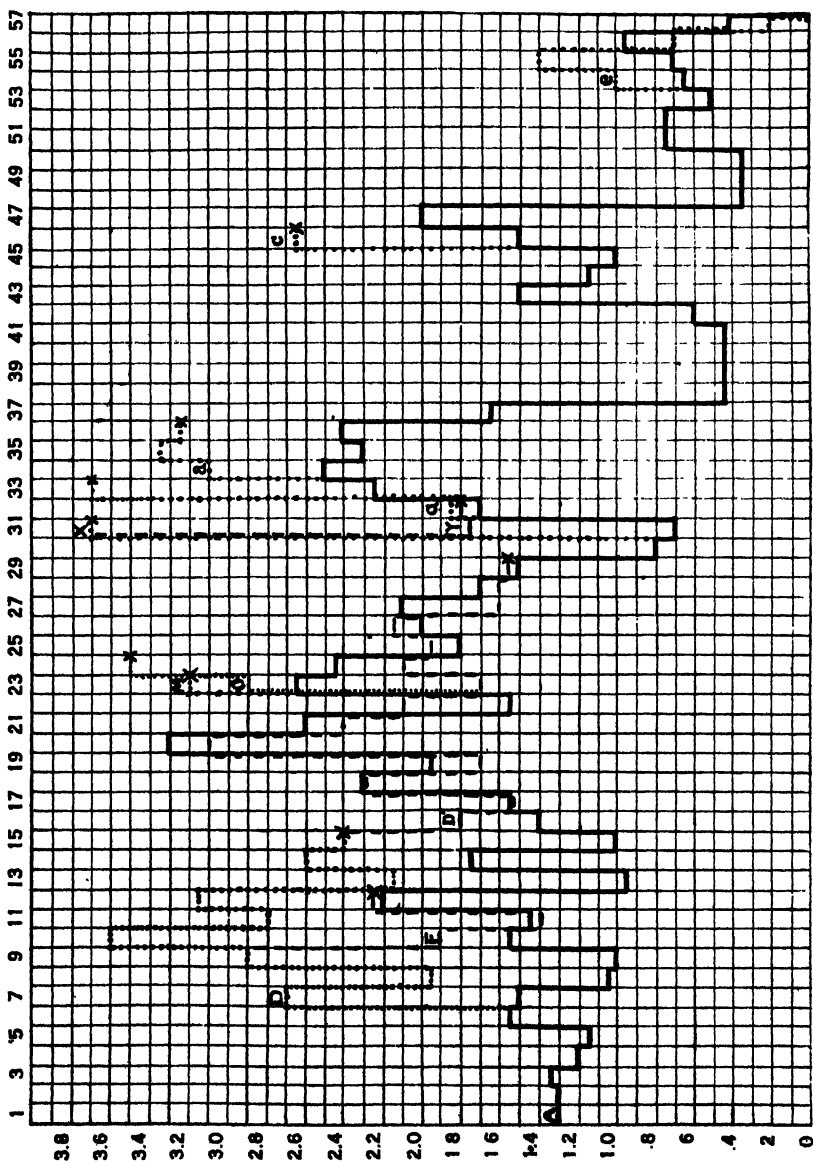


Diagram 2

treated in the same way except that thymus was substituted for thyroid. The table above shows the average division rate for each line during the periods in which the experiment was continued. As is seen, the average for D is always greater than for A, while E does not show this increase. The F line, started from the D line after fifteen days of thyroid treatment and kept thereafter in control medium, shows a return to the average rate of A.

Experiment II. April 24 to May 8, 1913.

Average Division Rate

PERIOD	G (CONTROL)	H (THYROID)	J (THYMUS)	A (CONTROL)	D (THYROID)	LINE
10	2.15	3.15	1.00	1.55	3.60	
11	1.20	2.80		1.45	2.80	
12	2.50	3.10		2.20	3.15	

This experiment was commenced during Experiment I to discover whether the effects observed in the thyroid treated individuals would be produced in other individuals of the same race. An individual was selected from the A line, and allowed to divide until there were twelve cells of the same generation. Four of these were allowed to continue in the control medium, forming line G, four were treated with Thyroid III (H), and four with Thymus III (J). The table shows the division rate for the three ensuing periods. For purposes of comparison, I have added the averages given by the control and thyroid lines of the preceding experiment for the same periods. This experiment confirms the results of Experiment I.

Experiment III. June 28 to July 2, 1913.

Average Division Rate

PERIOD	A (CONTROL)	M (THYROID)	N (THYMUS)	LINE
23	2.65	3.20	2.15	

This experiment was performed at Woods Hole. After the trip from New York and the change to Woods Hole tap water

the division rate as a rule averaged much higher than it had in the city. As my supply of Thymus and Thyroid IV was limited, and owing to the difficulty of keeping fresh tissue, this experiment was continued for one period only. It is sufficient however to show the typical thyroid increase. Compare the following experiment.

Experiment IV. June 28 to July 7, 1913.

PERIOD	Average Division Rate		LINE
	D' (CONTROL)	O (THYROID)	
23	1.70	2.90	.
24	2.10	3.50	

This experiment was carried on at the same time as Experiment III. The control in this case was the D line (now known as D') which had been treated with thyroid for 9 periods and then maintained on a control medium since the close of Experiment I. While in this medium it had approximately given the same averages as A, as may be seen from diagram 2. The O line was derived from this and treated with Thyroid IV for two periods, during which, as may be seen from the table, it maintained a much higher rate of division.

Experiment V. August 7 to 21, 1913.

PERIOD	Average Division Rate				LINE
	A (CONTROL)	X (THYROID)	Y (THYROID-CONTROL)	Z (THYMUS)	
31	0.70	3.70	1.75		
32	1.70		1.80	1.80	
33	2.25		2.50	2.00	

At the time of this experiment the life-cycle shows an apparent depression period (diagram 2) and the race was reduced to two individuals. One of these was kept in the control medium to continue the A line, the other was treated with Armour's desiccated sheep thyroids (Thyroid V) to start the X line. After

DISCUSSION

In the experiments described above, we have seen that in every case, disregarding for the moment Experiment IX, the thyroid treated line has divided more rapidly than the control line from which it was descended. Thymus treated lines on the contrary, while apparently showing a decreased rate of division, have in reality given only negative results, for the lower rate of division was caused by a greater number of deaths. Lines returned to the control medium after several periods of thyroid treatment have then given the same average division rate as the control, while their sister lines under continuous thyroid treatment have continued at the higher rate of division.

The source of the glands used bears no relation to the effects produced. A typical thyroid increase was produced by preparations from fresh glands of rats, rabbits and cattle, and from dried glands of sheep. The stability of the agent is shown by the fact that emulsions have continued active for twenty-five days during which time putrefactive decomposition had commenced. The accompanying graph (diagram 3) shows the effect of two emulsions (Thyroid II and III) the one from rats, the other from rabbits, for five successive periods. It will be seen that the two curves are similar, although at this time I have not sufficient data to discuss the nature of the curve.

I was however unable to secure any positive results with filtered extracts, either from fresh or dried material. This is the reverse of the results of Nowikoff ('08) who has published the only other experiments on this question I have been able to discover in the literature. It might be well then to discuss this paper in some detail.

While studying the chemotaxis of *Paramecium* to thyroid extract, Nowikoff observed that the individuals treated with thyroid seemed to divide more rapidly than those kept in hay infusion. He devised some experiments intended to demonstrate this increase in division rate: from these he concluded that the thyroid extract "exerts an intensive influence." Nowikoff used Merck's *Glandula Thyroidea sicc. pulv. dissolved in*

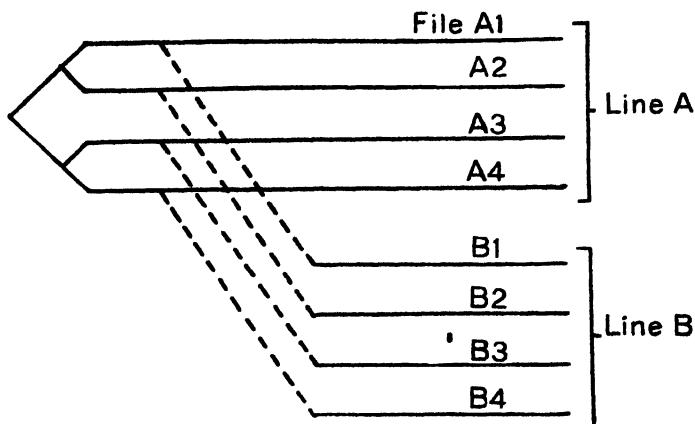


Diagram 3 Chart showing comparative effects by five day periods of Thyroid II and Thyroid III. The ordinates represent the increase of the thyroid average over the control average per period, the abscissae the periods covered.

———— = Thyroid II

. = Thyroid III

distilled water, shaken and filtered two or three times through paper, in a one or two per cent solution. A number of individuals (one to three) were placed in a few drops of this extract on a slide; other individuals were placed in a similar quantity of hay infusion or other media. The preparations were covered with a cover slip and set aside in a moist chamber. Daily observations were made and showed larger numbers of individuals in the thyroid media than in the others.

This method of Nowikoff's does not seem, however, adapted to answer the question at issue. Woodruff's experiments ('11) upon the toxicity of the material excreted by *Paramecia* have shown that the division rate of individuals kept in the same medium for a number of days grows slower owing to the harmful effects of this excreted material. It is significant that in Nowikoff's experiments the *Paramecia* kept in hay infusion under these conditions died out in a few days, as we should expect. Then too the thyroid extract, kept in a moist and fairly warm chamber for periods up to a month, must have undergone appreciable chemical change. It is evident that the experiment is complicated by the introduction of a toxic, if not lethal, factor and by the lack of a uniform thyroid preparation.

Another source of error in these experiments lies in the absence of an attempt to secure closely related *Paramaecia* for the experiment, the necessity for which is shown by the great variability in reaction and viability possessed by different races and individuals of this form.

I have endeavored to calculate from Nowikoff's daily counts of individuals the number of divisions which took place, although as I have pointed out this is made difficult by our ignorance of the number of deaths which occurred. This calculation is shown below, and indicates that using the rate of division as an index, his results are inconclusive.

Calculated division rate for Nowikoff's Paramaecia treated with Thyroid, Hay Infusion, and Muscle

MEDIUM	DAY										TOTAL
	1	2	3	4	5	6	7	8	9	10	
Hay Infusion..	0	0	0	0							0
Thyroid....	0	3	3	0	0						6
Thyroid..	0	3	2	1	0						6
Muscle....	0	2	1	0	2	0	0	0	1	0	6
Hay Infusion	0	0	3	1	0	0	0	1	0	0	5
Thyroid.....	3	3	1	0	0	0	0	0	0	0	7
Thyroid.....	2	1	0	3	0	0	0	1	0	0	7

Gudernatsch, in his second study of feeding experiments on tadpoles ('13), describes an experiment in which small pieces of thyroid gland were placed "in a small glass vial closed with gauze and allowed to stand upright, the open end being under the surface of the water." The typical rapid macroscopic differentiation normally produced by thyroid feeding was observed, and Gudernatsch concludes that "the product of the thyroid which caused the rapid differentiation must be soluble in water." The conditions of the experiment do not however preclude the possibility of minute particles of the thyroid escaping through the gauze and becoming food for the tadpoles. A definite conclusion can only be drawn from experiments conducted with carefully filtered preparations.

In order to secure a conclusive answer to the question whether *Paramecia* actually ingested particles of thyroid, the writer performed the following experiment, suggested by the methods employed by Métalnikow ('12). Having stained some particles of Armour's desiccated thyroids with an alcoholic solution of Congo Red, and washed out the free color in distilled water, they were placed in a watch-glass of boiled tap water with several individuals of *Paramecium caudatum*. It was possible to watch the formation in the *Paramecia* of gastric vacuoles containing these prepared thyroid particles, and, following the cyclosis, to observe the change from acid to alkaline reaction shown by the Congo Red as described by Métalnikow. I have permanent preparations fixed with osmic acid vapor that show these thyroid filled vacuoles very clearly.

If then the *Paramecia* can and do ingest these thyroid particles and if after ingestion the chemical reactions which are commonly accepted as indicating digestion are observed, does it not follow that the effects produced by the thyroid result only after ingestion and presumably digestion? Certainly this is in accord with Gudernatsch's results.

The individuals treated with thyroid were as a rule more active, and slightly smaller than the control individuals, and their protoplasm was more transparent. This would be the case if the process of metabolism were accelerated by the thyroid. The thymus treated individuals on the other hand were more sluggish, larger and darker than the control forms, these appearances apparently being due to large quantities of undigested food granules. The conclusion seems obvious that the effect of the thyroid is directly upon the metabolic activities of the cell.

With the effects of the thyroid upon growth and differentiation this investigation is obviously not concerned.

Returning now to Experiment IX we find the data presented more difficult to analyze. Here the thyroid does not increase the division rate at a time when the control line is losing the power of division and evidently dying out. Compare this with Experiment V, when the control line was also at a very low state of

vitality, but the thyroid treatment resulted in the greatest increase in division rate observed at any time.

The explanation, I believe, lies in the fact that in these two Experiments the *Paramaecia* were at very different stages in their life history and that their protoplasmic make-ups were not comparable. I believe that in Experiment V a typical depression period (Calkins) or rhythm (Woodruff) was occurring, from which the race was able to recover without stimulation, while the thyroid was able to produce its usual effect. In Experiment IX however I suggest that we have a period of depression leading to "germinal death", such as carried off Calkins' race of *Paramaecium caudatum*. Under this hypothesis we can understand how the thyroid was without effect in the final depression period, for if some element of the protoplasm necessary for digestion were lacking, the thyroid agent could not be released or act. While this is advanced only as an hypothesis, it is a significant fact that at the time when the race was nearing the end of its life cycle, the thyroid which had previously produced a significant increase in the rate of division with such absolute uniformity, presented no perceptible effect. Attention is also called to the fact that this race of *aurelia* died out in four hundred and twenty generations, while Woodruff's race of the same species has been maintained for more than four thousand generations, one more example of the great differences in vitality manifested by different races of *Paramaecia*.

SUMMARY

Experiments have been carried on to demonstrate the effect of thyroid and thymus added to the diet of a pedigreed line of *Paramaecium aurelia* during a life history of four hundred and twenty generations. These experiments have shown that the effect of the thyroid is to increase greatly the rate of division, except at the time when the line was nearing the close of its cycle; and further that the effect is not permanent after feeding with thyroid is stopped. Similar treatment with thymus gave negative results. It has been shown further that the *Paramaecia* actually ingest and probably digest particles of thyroid.

From these experiments it has been suggested that the effect of the thyroid is primarily upon the metabolic activities of the cell, and further, that at the close of the life cycle some element of the protoplasm necessary to digestion is lacking, thus inhibiting the effect of the thyroid.

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APPENDIX

Daily records of divisions

Here are given the number of divisions per day for each file of every line described in Experiment 1 of this report (the other experiments are only summarized in the preceding pages). At the end of every five days the total number of divisions for each file for that period is given. The averages cited in the text are obtained by adding the totals of the files of each line and dividing by 20 (or the exact number of opportunities for division).

A letter and number enclosed in brackets are used to designate from what file an individual has been replaced after its loss from death or accident, e.g. (A2). The symbol ° is used to indicate that an individual died but was replaced by a sister individual from the last preceding division of the same file. In this case the number of divisions of the sister individual are credited to the file, so 1°. The symbol — indicates a death without replacement.

Experiment I

APRIL	9	10	11	12	13	(7)	14	15	16	17	18	(8)
A1	0	2	2	2	1	7	2	2	1	0	(A2)	5
A2	1	1	2	1	2	7	2	1	0	(A3)	1	4
A3	0	1	1	0	0	2	0	0	(A4)	1	1	2
A4	0	2	2	2	2	8	2	2	1	1	1	7
D1	(D3)	2	4	3	3	12	3	1	2	1	2	9
D2	(D4)	2	4	3	3	12	2	4	1	2	2	11
D3	2	2	3	3	3	13	2	3	0	1	2	8
D4	1	2	4	3	2	12	3	2	2	2	2	11
E1	0	2	1	2	(E2)	5	(A1)	(E3)	(E3)	1	2	3
E2	0	1	1	(E1)	2	4	(A1)	(E3)	1	(E3)	1	2
E3	0	(E1)	0	(E4)	—	0	(A2)	2	2	2	1	7
E4	0	1	(E1)	1	—	2	(A2)	(E3)	2	2	1	5

Experiment I (continued)

APRIL	19	20	21	22	23	(9)	24	25	26	27	28	(10)
A1	1	1	1	2	0	5	1	2	2	1	2	8
A2	1	1	1	2	1	6	1	2	1	1	2	7
A3	2	1	1	1	0	5	2	2	2	1	2	9
A4	1	1	2	0	0	4	0	(A3)	2	2	3	7
D1	4	3	2	3	2	14	4	4	3	3	0	14
D2	4	2	3	3	2	14	4	4	4	4	5	21
D3	3	3	3	3	3	15	1°	3	4	4	5	17
D4	3	4	2	3	3	15	3	4	4	4	5	20
E1	1°	1°	(E3)	2	—	4	F1	3	0	2	1	(F2) 6
E2	1°	1°	(E3)	3	5	F2	3	3	2	3	2	13
E3	1	1°	2	2	1	7	F3	2	2	3	2	10
E4	1°	1	(E3)	3	—	5	F4	2	2	1	3	9

APRIL	29	30	MAY 1	2	3	(11)	4	5	6	7	8	(12)
A1	1	1	1	2	2	7	3	2	1	3	1	10
A2	1	1	1	3	1	7	3	3	1	3	1	11
A3	1	(A4)	2	2	2	7	2	3	2	2	2	11
A4	1	1	2	2	2	8	3	3	1	3	2	12
D1	0	(D2)	4	3	4	11	4	3	2	4	2	15
D2	3	1	4	4	3	15	4	3	2	4	2	15
D3	3	2	3	4	3	15	5	3	3	3	2	16
D4	3	2	3	4	3	15	5	3	3	3	3	17
F1	1	0	2	2	2	7	4	3	1	3	1	12
F2	2	1	2	0	2	7	3	2	2	3	1	11
F3	2	1	1	1	2	7	2	2	2	3	1	10
F4	2	1	1	1	2	7	3	3	2	3	1	12

* MAY	9	10	11	12	13	(13)	14	15	16	17	18	(14)
A1	2	0	(A2)	0	2	4	2	3	3	1	2	11
A2	1	0	1	0	2	4	2	0	1	2	2	7
A3	2	1	0	1	2	6	1	2	2	1	2	8
A4	1	1	0	1	2	5	1	2	3	1	2	9
D1	3	2	2	1	2°	10	3	3	3	2	2	13
D2	3	2	2	1	3	11	3	3	3	2	2	13
D3	3	2	1	2	3	11	3	3	3	2	2	13
D4	3	3	1	1	3	11	3	3	3	2	2	13

Experiment I (continued)

MAY	19	20	21	22	23	(15)
A1	1°	1	1	1	1	5
A2	2	0	1	1	1	5
A3	1	2	0	1	1	5
A4	2	0	1	1	1	5
D1	4	3	2	1	1	11
D2	4	3	2	2	2	13
D3	3	3	1	2	3	12
D4	1°	(D3)	2	1	0	4

A THIRD SEX-LINKED LETHAL FACTOR IN DROSOPHILA

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THREE FIGURES

In an experiment (reported in the *Jour. Exp. Zoöl.*, page 390, November, 1911) in which three sex-linked characters were involved, namely, white eyes, vermilion eyes and miniature wings, certain results were obtained involving double crossing-over. The more recent results indicate that double crossing-over was excessive in this experiment.¹ A white miniature male had been mated to a vermilion pink ('orange') female. The daughters were wild type (red, long) and the sons vermilion (long). The two sex-chromosomes of these daughters contain respectively the genes for white (w), red (V), and miniature (m); and for red (W), and vermilion (v), and long (M). All later work has shown the order of the genes here involved is white, vermilion, miniature, as shown in diagram 1.

On this basis, the results of the previous experiment in terms of cross-overs would be as follows:

	<i>Total</i>	<i>Per cent</i>
Non-cross-overs (vermilion and ver. pink males)	333	74 7
Single cross-overs between white and vermilion (min. and min. pink males).....	81	18.2
Single cross-overs between vermilion and min. (verm. min. and ver. min. pink males).....	11	2 4
Double cross-overs (wild type and pink males)...	21	4 7

Instead of giving fewer double cross-overs than singles, according to expectation, the number of doubles was twice that of singles, and for numbers as large as these this difference cannot be due to

¹ In the analysis on page 392 the large C in 'sperm no X' should be small c. This change adds eight classes to the white males and eliminates the same number from the colored classes. The expected numbers in the male classes at the top of page 393 should read downwards: 3-3-1-1-8-3-3-1-1-8.

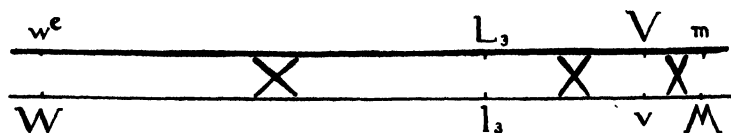


Diagram 1

chance. This experiment has been repeated, therefore, in small mass cultures and has given, in F_2 , the totals shown in table 1.

TABLE 1
Females

WILD TYPE	VERMILION	PINK	VERM. PINK
1428	1465	469	548

Males

VERMILION	VERM. PINK	MIN.	MIN. PINK	VERM. MIN.	VERM. MIN. PINK	WILD TYPE	PINK	WHITE	WHITE MIN.
501	161	251	84	14	35	7	0	314	1166

The females conform to expectation. The males show many irregularities that will be discussed in another connection. Classified as in the original experiment, the males give:

	<i>Totals</i>	<i>Per cent</i>
Non-cross-overs.....	662	63.8
Single cross-overs between white and vermilion..	335	31.8
Single cross-overs between verm. and minia- ture.....	49	4.7
Double cross-overs.....	7	0.7

The totals of these new results for the males approach very closely to expectation both for the amount of double crossing-over and also for the distances apart of the genes, despite other inequalities that appear in the results.

In the experiment as actually carried out a non-sex-linked gene, namely, the gene for pink eyes, is involved. The additional classes that are thus introduced are ignored in the preceding calculations in which pink and not-pink classes are combined. If we take pink into consideration the following relations will be found. The gene for pink lies in another chromosome, and since the F_1

female is heterozygous for pink every egg after the polar bodies are extruded will contain either the pink factor or its normal allelomorph. The male has two classes of sperm, viz., one with and the other without the pink factor. In all cases except white (which remains white in external appearance in either case) the combination gives two classes in the ratio of 3:1. The relation of pink to not-pink are everywhere normal except in the following case. The single cross-overs between vermilion and miniature are vermilion miniature (14) and vermilion miniature pink (35). Instead of the expected 3:1 ratio the relation is almost reversed. I can offer no explanation of the relative deficiency in the vermilion miniature class or the relative excess of the vermilion miniature pink.

We may assume that the double cross-over class included in white miniature is very small and negligible; if so, then the non-cross-over class white miniature should equal the sum of the vermilion and vermilion pink, which is 662. In fact, it is nearly double (1166) that number. This disturbance, as will now be shown, is due to a lethal factor present in the stock.

THE LOCATION OF LETHAL III

In the experiment, made as explained above to test double crossing-over, only about half as many males as females appeared in some of the cultures. This kind of result we have come to associate with a lethal factor, and further tests made with some of the F_2 females show, in fact, the presence of a new lethal. Table 2 gives individually the ten original mass cultures (p. 318).

Since the cultures were made in mass they can not be used to study lethal factors, for, the offspring may have arisen from both lethal-bearing and non-lethal-bearing females. The high sex-ratio was not noticed until the experiment was nearly finished, and then it was possible to obtain only a few females.² In three cases

² We have known that non-disjunction occurs in several of our stocks, notably in the white. It occurred in the mother in the experiment just described, for when vermilion pink females were mated to white miniature males there were produced:

Verm. ♀ 2	Red ♀ 92	Verm. ♂ 46	White ♂ 2
--------------	-------------	---------------	--------------

The two vermilion females and the two white males must have arisen through

TABLE 2

Females

	WILD TYPE	VERMILION	PINK	VERMILION PINK
I.....	128	123	39	51
II.....	151	154	37	58
III.....	173	169	61	76
IV.....	86	80	37	36
V.....	250	231	51	81
VI.....	84	84	41	44
VII.....	90	93	32	33
VIII.....	94	96	28	31
IX.....	189	228	68	58
X.....	184	188	75	80
	1428	1465	469	548

Males

	VERM.	VERM PINK	MIN.	MIN. PINK	VERM. MIN.	VERM MIN. PINK	WILD TYPE	PINK	WHITE	WHITE MIN
I..	57	16	24	9	3	10			33	120
II.....	72	25	30	5	2				36	142
III.....	24	15	29	9	1		2		29	133
IV.....	26	4	10	2	1		2		16	37
V....	103	30	46	17	2	11			54	193
VI.....	34	12	11	7		2	1		16	78
VII.....	16	5	12	5					13	66
VIII.....	35	4	17	10	1	7			17	74
IX.....	63	24	28	10	2	1	1		45	173
X....	71	26	44	10	2	4	1		55	150
	501	161	251	84	14	35	7		314	1166

at least, viz., IV, VIII and X, the sex-ratios were again obtained from F_2 females. Further tests were made of the stock derived from the F_2 lethal females in order to locate the lethal or lethals present.

non-disjunction. One of the vermilion females was tested by breeding to a pink male. She gave:

Red ♀ 56 Pink ♀ 61 Verm. ♂ 43 Verm. pink ♂ 19 Red ♂ 2

The two red males are again due to non-disjunction.

Vermilion females were out-crossed to ♂ club males. From those sets that gave a sex-ratio, daughters (red eyed) were mated singly to their vermillion-eyed brothers. About half of the females gave no sex-ratios, as follows:

All females	Club ♂	Verm. ♂	Club verm. ♂	Wild type ♂
687	219	314	58	64

The cultures that gave sex-ratios follow (table 3):

TABLE 3

ALL FEMALES	CLUB ♂	WILD TYPE ♂	CLUB VERM. ♂	VERM. ♂
146	53	3	1	
176	59	7	3	
145	34	10	2	1
121	36	8	5	
588	182	28	11	1

The smallest class of males is vermillion, which we assume therefore to be the double cross-over class. Since the lethal went in with the vermillion female one of the chromosomes of her daughter will contain the genes for lethal and for vermillion; and the homologous sex chromosome will contain the gene for club. The relation of the two chromosomes with the normal allelomorphs of these genes are shown in diagram 2.

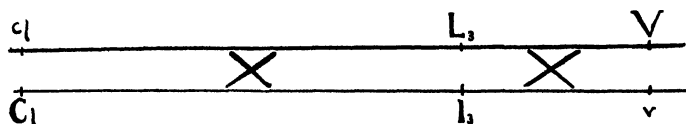


Diagram 2

In order that vermillion become the double cross-over class the lethal must be between club and vermillion, as in the diagram. The chromosome distances calculated from the results are as follows:

Club lethal III.....	13.0
Lethal III vermillion.....	5.4
Club vermillion.....	18.0

Other vermilion females were out-crossed to eosin miniature males. From the cultures that showed sex-ratios females were back-crossed singly to eosin miniature males. The back-cross cultures that gave sex-ratios are seen in table 4.

The results of the experiment confirm the location of the genes in diagram 1, as the following chromosomal distances show:

Eosin lethal III....	20.2
Eosin vermilion.....	27.0
Eosin miniature.....	29.0
Lethal III vermilion..	7.0
Lethal III miniature..	9.3
Vermilion miniature..	2.3

TABLE 4
Females

EOSIN MIN.	WILD TYPE	EOSIN	MINIATURE
78	104	33	24
90	89	36	33
82	84	37	45
101	77	31	30
105	97	31	43
127	86	35	33
62	62	29	13
60	53	36	38
58	51	24	30
763	703	292	289

Males

EOSIN MIN.	MINIATURE	EOSIN VERM.	EOSIN	VERM.	WILD TYPE
84	18	8	4		
101	20	6	2		
96	27	11	1	1	
95	27	3	3		
103	17	11	3		
128	16	13	2		
52	17	2	2		1
76	31	10	3		
39	23	7	2	1	
68	26	8	4		
52	19	6	4		
44	24	6			
938	265	91	30	2	1

The small numbers in the first experiment gave a distance between lethal III and vermilion of 5.4; the second gave 7. The data of both experiments gave 6.8.

In a third case vermilion females (lethal-bearing) were crossed singly to vermilion miniature males and gave:

VERMILION ♀	VERM. MIN ♀	VERM. MIN. ♂	VERMILION ♂
113	78	75	7
77	99	79	7
190	177	154	14

These results gave a distance of 9 units between the lethal involved and miniature and the lethal may lie either to the right or to the left of miniature. If to the left, the locus corresponds to lethal III and may be so taken.

A HIGH SEX-RATIO

A vermilion female (IXX^a) second generation from F₂ was bred to a vermilion male. Two of her vermilion daughters were bred (in the same culture)^a to two white vermilion brothers and gave a ratio of:

Vermilion ♀	Vermilion ♂
71	3

A remarkable high sex ratio is here met with. Many of the vermilion daughters were bred separately. A partial report of the results, namely, most of the cases in which the females were bred to eosin miniature males, is included in table 5.

Certain other daughters (of the 71) were bred to their (three) vermilion brothers and gave the results shown in table 6.

Still other daughters (of the 71) bred to eosin miniature males gave the results seen in table 7; where, in addition to the regular classes of females, there are some irregular classes that cannot be explained. These exceptional individuals appeared only in the

^a It is possible that the offspring came from only one of these females.

TABLE 5

WILD TYPE ♀	WHITE-EOSIN COMPOUND ♀	WHITE ♂	VERMILION ♂	PER CENT OF CROSS-OVERS
59	43	37	7	16
35	44	38	6	14
70	81	50	15	23
48	38	31	13	30
71	63	62	17	22
48	57	53	16	23
68	65	71	16	18
87	95	68	27	28
59	34	38	9	19
61	60	45	8	15
86	66	52	15	22
61	59	63	16	20
23	35	14	0	
70	62	60	11	15
43	60	50	10	17
40	53	30	10	25
51	49	33	9	21
52	51	23	4	15
53	51	40	4	11
1085	1066	858	213	

TABLE 6

VERMILION ♀	WHITE ♂	VERMILION ♂	PER CENT OF CROSS-OVERS
184*	78	19	19
62	35	5	12
79	28	4	13
25	8	3	27
83	20	4	17
56	21	5	19
489	190	40	

*Mass culture which is not included in curve on page 323.

TABLE 7

IRREGULAR ♀ ♀		REGULAR ♀ ♀				PER CENT OF CROSS-OVER
White ♀	Verm. ♀	Red ♀	White-eosin compound ♀	White ♂	Vermilion ♂	
	3	32	26	36	10	22
11	16	51	41	53	10	16
7	4	38	15	21	2	9
13	22	14	38	27	9	25

early hatches of the cultures.⁴ On the other hand, the proportion of males is the same as in the other cultures.

In the last columns of the three preceding tables the percentages of cross-overs between white and the lethal are given. If these are tabulated they show bimodal curves (diagram 3).

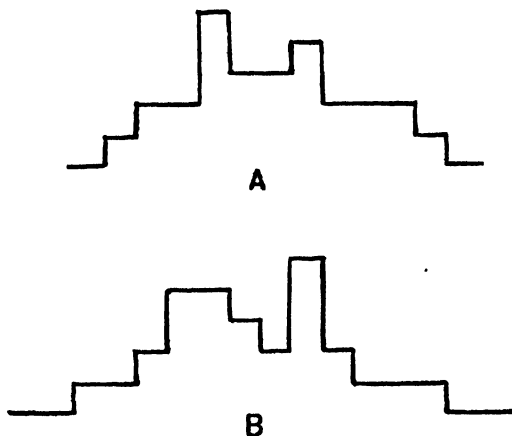


Diagram 3 Bimodal curves from the grouping of the linkage values from tables. A, grouped 9 + 10, 11 + 12, etc. B, grouped 8 + 9, 10 + 11, etc.

All the data taken together suggest the following interpretation. If an original female that gave rise to the 71:3 ratio had two lethal genes that are not allelomorphs, one in each of her sex chromosomes, all of her sons should perish except those few which resulted from crossing-over. The three males would be these cross-overs and indicate the amount of crossing-over that had taken place (provided their number is doubled, for the other equal class of cross-over males perished). Using the 71 females as the number of all the expected males and dividing this into 6 would give the percent of cross-overs, namely, 8 per cent.

In the second place, all the 71 daughters should be lethal-bearing, except 8 per cent corresponding to the males that survive. *In fact, all of 34 females tested were lethal.*

⁴ If the first-hatched daughter had been fertilized by the father before they were removed (as virgins!) this result can be explained. In the next generation no repetition of the occurrences was seen.

In the third place, the linkage values of these daughters should fall into two classes corresponding to the two lethals, and since the two lethals are only 8 units apart the curve representing the values should have two modes about 8 units apart. This result also seems to be realized, as shown in diagram 3.

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A GENE FOR THE FOURTH CHROMOSOME OF DROSOPHILA

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Drosophila ampelophila contains two pairs of long 'autosomes' (chromosomes other than sex-chromosomes) and one pair of minute 'autosomes'; there are, in addition, a pair of long sex-chromosomes alike in the female ('X's) but unlike in the male ('X' and 'Y').¹ Correspondingly, breeding tests of the numerous mutants of *Drosophila* have revealed a great group of genes (containing over thirty members) which are sex-linked in such a way that they are distributed, in the reduction division, to exactly the same cells as is the X-chromosome, and two other great groups (with about twenty members each) which are not sex-linked and so have been considered to follow in their distribution two of the pairs of autosomes (presumably the long ones). The genes in these three groups are therefore said to lie in Chromosomes I, II and III, respectively. The members of any group of genes are linked with one another; in the female the linkage is partial and is of the linear type to be expected on the basis of the chiasmatype theory of Janssens and Morgan; in the male the linkage is complete (no crossing-over). All the members of one group assort independently of the members of either of the other groups, in both sexes, as would be expected on the generally accepted postulate of the random assortment of non-homologous chromosomes in the reduction division.

¹ Two lines of recent cytological investigation, the one followed by Mr. C. W. Metz, the other by Mr. C. B. Bridges, have given evidence (not yet fully published) which goes far towards refuting the view suggested by Stevens that the so-called 'X' chromosome of *Drosophila* actually consists of a short autosome with the real X-chromosome joined to it and that the 'Y' chromosome represents this short autosome without any 'X' attached.

It will be seen that the above grouping of genes leaves one pair of autosomes (presumably the small ones) and the Y-chromosome without any genes to correspond. As regards the Y, it seems at first sight surprising that no mutations have been found involving genes which follow it in their distribution (i.e., genes which are always transmitted from father to son), for it is of about the same length as the X, in which over thirty genes are known. This fact would force us to one of three conclusions: (1) Either the genes connected with the Y-chromosome for some reason do not mutate; or (2) these mutations are all recessive to dominant normal allelomorphs present in X (in spite of the fact that mutations in X are not dominated, conversely, by allelomorphs in Y); or (3), as Mr. C. W. Metz first suggested, genes are degenerate or entirely absent in the Y-chromosome. The first conclusion is *a priori* unlikely; the third may now be supported by a number of considerations, which it will be of interest to discuss briefly in this connection, before proceeding to our account of the fourth chromosome.

In the first place, the Y-chromosome is known to vary greatly in size and number in closely related species of animals. Secondly, as Mr. C. B. Bridges has recently shown, a female occasionally, owing to an abnormal reduction division ('non-disjunction'), receives a Y-chromosome in addition to the two X's, yet such a female is indistinguishable from the ordinary form, which contains no Y. The Y-chromosome therefore either contains no genes or else only genes which are allelomorphic to those in X, but never dominant to them. This is proved also by the fact that mosaic flies sometimes develop, of which a part of the body is female but another part is male, owing to the accidental loss of one of the X-chromosomes in an embryonic cell-division. These male parts must have an X, but no Y, and yet they are indistinguishable from corresponding parts on real males which contain a Y. Furthermore, Mr. C. B. Bridges has obtained (again by 'non-disjunction') males which must have two Y's, yet these also show no peculiarities. It is therefore certain that Y either contains no genes at all which have an effect upon the individual or it contains genes allelomorphic to those in X. It can be proved, however, that if it contains allelomorphs to the

genes in X, these are not normal allelomorphs (i.e., the same kind as those present in the X of the wild fly) for they are recessive to mutant genes in X to which the normal genes in X are dominant, i.e., a male always manifests all the genes, mutant or normal, recessive or dominant, that are present in its single X-chromosome. In other words, if there be genes in Y allelomorphic to those in X, they are abnormal allelomorphs of those in X, and are always recessive to all genes in X. It is difficult to conceive why genes in Y should be recessive, on the one hand, to normal genes in X, in those cases where the mutant genes in X are dominant to the normal, and, on the other hand, to mutant genes in X, in those more frequent cases where the normal genes in X are dominant to the mutant, unless the genes in Y are mere 'absences' or nearly so.²

There is an *a priori* explanation for this lack of genes, or lack of dominant genes, in the Y-chromosome, an explanation in the development of which Dr. A. H. Sturtevant has coöperated with me. Owing to the fact that crossing-over never occurs in the male *Drosophila*, any mutation which originally occurred in Y remained in that chromosome and was never exchanged for a normal gene from X. Furthermore, these mutations in Y which were recessive would not have been subject to the action of natural selection for, since the normal gene in the X-chromosome will dominate over them, individuals containing them will not be abnormal. In the course of time, therefore, recessive changes in the Y-chromosome will tend to accumulate. If, now, we assume that mutations sometimes consist in losses³ of genes, a degeneration of the Y-chromosome (so far as its *genes* are concerned) would result.

² This might be otherwise explained on the very improbable hypothesis that all of the mutations which occurred in X were in a restricted part (the end?) of the chromosome, and that the Y lacked only this part of the chromosome and so appeared to contain genes recessive to all those in X which underwent mutation, although it actually contained normal genes in the other part of the chromosome, which never mutated. This is not quite equivalent, although nearly so, to the idea that X is attached to an autosome (see footnote 1).

³ By losses of genes are meant not necessarily their total bodily disappearance (losses of loci from the chromatin) but also any changes in them whereby they are rendered permanently inactive and incapable, under any circumstances, of exerting an influence on the organism.

This is because most losses of genes, if they occurred, would be recessive (i.e., one 'dose' of a factor usually has the same effect as two), as indicated by the fact that the one-X part of a mosaic is like the XX part in respect to all characters affected by X except the sex characters (provided the two X's of the XX part are alike). Now, if, as seems likely, recessive losses are more apt to occur than recessive additions of genes (since one 'dose' usually has the same effect as two) the Y-chromosome will gradually become functionless. On *a priori* grounds, we should expect such a fate, in any species, for the sex-chromosome peculiar to the heterozygous sex, if crossing-over never occurs between it and its homologue.⁴

These considerations will also explain the size difference which often exists between X and Y, the apparent unimportance of supernumerary Y-chromosomes in development, when these occur, and other irregularities of the Y-chromosome. Hitherto it has been a mystery why the difference merely in the sex factor or factors contained in the sex chromosomes should often be correlated with such a large difference between the two chromosomes, whereas differences in respect to other factors did not involve any visible size differences in the chromosomes concerned, which presumably contain hundreds of genes.

There is, therefore, all things considered, no cause for surprise in the fact that no mutations have occurred involving a group of genes transmitted only from father to son, and thus following in their distribution the Y-chromosome. The chief gap, if it may be so termed, then remaining in the parallelism between the configuration of the chromosomes and the distribution of genes in *Drosophila* has been due to the fact that no genes were found in a fourth independent group (a third independent non-sex-linked group) to correspond with the fact that there are three pairs of autosomes.

⁴ Conversely, where this chromosome appears degenerate or different genetically from its homologue (in addition to the difference in the sex factors) we should expect to find no crossing-over between the two sex-chromosomes in the heterozygous sex, i.e., complete, not partial, sex-linkage. This argument applies to an known cases of sex-linkage, for in all these cases a recessive mutant factor call manifest itself in the heterozygous sex, proving that the sex-chromosome peculiar to the heterozygous sex is different from the other sex-chromosomes in that it contains no dominant normal allelomorph of the mutant factor.

Supposing, however, that mutations are equally likely to occur at any locus in the chromatin, it could be explained as a result of pure chance that no mutations had as yet happened to lie in the restricted space of the small autosome. It was expected, nevertheless, that mutations in a fourth group would eventually be found, and such a mutation has now in fact arisen.

The new character is a recessive wing and leg abnormality, the wings being held out from the body but bent backwards near the base, and the metatarsal joint of the legs being frequently greatly shortened and thickened. The wing is also apt to be curved, with the dorsal surface convex, and shortened. The character varies somewhat, but there is very rarely any difficulty in distinguishing it from the normal form, unless the flies have been raised in very dry bottles. Drought therefore hinders the manifestation of this character, as it may also, and to a greater degree, in some way hinder the development of the character 'abnormal abdomen,' a case reported by Morgan.

I found the mutation 'bent wing' in a race with bifid wings and vermilion barred eyes, all three of these characters being in Chromosome I (sex-linked). A cross of bifid vermilion barred bent male by a pink-eyed female (pink is in Chromosome III) gave in F_1 all the males normal, and the females also normal except in respect to barred, which is dominant; in the next generation, F_2 , all combinations of the factors concerned appeared. If bent had been in Chromosome III, no crossing-over would have occurred between bent and pink in the F_1 male, consequently, no pink bent spermatozoa could have been formed, and thus (since pink and bent are both recessive) no pink bent F_2 individuals could have been produced. Since these were produced, bent did not lie in the *third* chromosome. Similarly, if bent had been in the first (X) chromosome or in the Y, no crossing-over between it and the sex-factor would have taken place in the P_1 or F_1 male, and consequently no bent females could have been produced in F_2 . As bent females were produced, bent did not lie in the *first* (X) chromosome, or in the Y.

A bent barred eyed F_2 male was then crossed to a female containing three mutant genes in the *second* chromosome, namely,

the genes for black body color, purple eyes, and curved wings. Here, too, some flies were obtained in F_2 which showed the characters of both grandparents at once (i.e., were both bent, and black, purple, and curved, and sometimes also barred). This proved that bent did not lie in Chromosome II. The details of the count are shown in table 1.

TABLE 1

Not bent

WILD TYPE	BLACK PURPLE CURVED	BLACK	PURPLE CURVED	CURVED	BLACK PURPLE
84	30	1	3	6	5
<i>Bent</i>					
43	9	1	1		

The number of barred and non-barred, also of males and females, in each class of moderately large size, were approximately equal. The linkage manifested between black, purple, and curved corresponds as closely with expectation based on the previous linkage results of Bridges, as could be demanded for the small numbers involved. The determination of curved was at times uncertain, owing to the tendency of bent to curve too, and the determination of purple in eyes which were barred was also sometimes uncertain. But as far as the results go, they show that bent is independent of black, purple, curved, barred, and sex.

As no counts had been made in the cross with pink, and few were obtained in the cross with black purple curved, it was still conceivable that although bent was not absolutely linked, in the male, with the members of one of the three previously known groups, as has always been found to be the case with other genes, still it might perhaps be partially linked, in either or both sexes. New crosses were therefore made with the object of securing accurate counts. Some bent males descended from F_2 of the cross with pink were mated to black pink females. These males were found to be heterozygous for pink, as half of the F_1 flies were pink (although none were black or bent). The red-eyed F_1 flies were

mated together, to show the distribution of bent with respect to all three previously known chromosomes. The pinks were also mated together. The latter cross should show the distribution of bent with respect to Chromosomes I and II only, as all the flies were homozygous for pink, the mutant gene in Chromosome III.

The composition of the pink flies used as parents, with the paternal and maternal genes which they contained, was as follows:

F ₁ male	Paternal genes	Gray	Pink	Bent
	Maternal genes sex	Black	Pink	Straight
F ₁ female	Paternal genes sex	Gray	Pink	Bent
	Maternal genes sex	Black	Pink	Straight

The count of offspring of this cross of pink flies resulted as shown in table 2.

TABLE 2
All individuals pink

		WILD TYPE	BLACK	BENT	BLACK BENT
F ₂ numbers observed	females	83	30	24	8
	males.	77	24	31	9
	Total . .	160	54	55	17
Total numbers expected on independence		162	54	54	18

Numbers of females expected = numbers of males expected.

The results conform with the theoretical expectation on the assumption of independent segregation between black, sex and bent.

The composition of the parents in the cross of red flies was as follows:

F ₁ male*	Gray	Red	Bent
sex	Black	Pink	Straight
F ₁ female	Gray	Red	Bent
sex	Black	Pink	Straight

* The paternally derived allelomorphs are on the upper line, the maternal on the lower.

The offspring were as seen in table 3.

TABLE 3

		WILD TYPE	BLACK	PINK	BENT	BLACK PINK	BLACK BENT	PINK BENT	BLACK PINK BENT
F ² numbers observed	females...	87	40	36	17	5	6	12	3
	males...	98	27	39	29	17	10	10	3
	Total ...	185	67	75	46	22	16	22	6
Total numbers expected on independence		185	62	62	62	20.6	20.6	20.6	6.9

Numbers of females expected = numbers of males expected.

A considerable differential viability came into play in these bottles, as is proved by that fact that certain 'contrary' or opposite classes which were necessarily produced in equal numbers at fertilization (no matter whether linkage was involved or not), gave rather different counts of adult flies (e.g., 206 females; 233 males; 97 pink straight, 62 red bent; which were the worst discrepancies). On allowing for these differences obviously due to viability, we find that there are no discrepancies due to linkage of bent with black or pink, for the average between black straights and gray bents (pink plus red), is to the black bents as 3:1, and the average between pink straights and red bents (gray plus black) is to the pink bents as 3:1, as expected on independence. These results therefore confirm those previously obtained in regard to the independence of black (Chromosome II) and bent; they show in addition, however, that bent is independent of pink (Chromosome III).

The distribution of bent with regard to black and pink was next determined separately for each sex, by means of back-crosses. A normal male was mated to black pink bent females. The F₁ males, which were heterozygous for all three factors, as well as for sex, were then mated to triply recessive black pink bent females. If the factors were independent, all classes in the next generation should be equal in number. The actual result was as shown in table 4.

TABLE 4

CLASSES	WILD TYPE	BLACK	BENT	BLACK BENT	PINK	BLACK PINK	PINK BENT	BLACK PINK BENT
females	29	28	23	30	16	14	15	9
males	25	22	22	17	14	18	16	15
Total	54	50	45	47	30	32	31	24

Here practically the only irregularity not due to chance is obviously caused by a deficiency (low viability) of pink flies of all classes, and by a lower viability of males than of females. The independent assortment of bent with respect to the other factors, and of the other factors with respect to each other, is best brought out (especially in a case involving differential viability) by a tabulation of the percent of cases in which any two pairs of factors, considered by themselves, underwent recombination in the formation of the germ cells of the heterozygous parent. Thus, in the case of sex and bent, the factors for sex and for bent in the heterozygous male parent were both derived from the mother, the Y-chromosome and the normal allelomorph of bent ('straight') both coming from the father. Yet in the segregation division by which the sperm were formed, a recombination occurred as frequently as a persistence of the old combination, so that as many eggs were fertilized by sperm bearing the sex factor and straight, or the Y-chromosome and bent, as were fertilized by sperm with sex and bent or Y and straight. As a result, straight females and bent males formed 50 per cent of the total number, as we should expect on the basis that the factors were in non-homologous chromosomes which were assorted independently. The other per cents of recombination were as follows:

	<i>per cent</i>
Sex-black	50.0
sex-pink	55.0
black-pink	50.5
black-bent	50.5
pink-bent	49.0

In a reciprocal cross some of the triply heterozygous F_1 females were back-crossed to triply recessive males. Here, too, we should

expect all classes equal, barring differential viability, on the basis of independent assortment, and approximately 50 per cent of recombination between any two factors. The result was similar to that obtained in the back-cross of the males (table 5).

TABLE 5

CLASSES	WILD TYPE	BLACK	BENT	BLACK BENT	PINK	BLACK PINK	PINK BENT	BLACK PINK BENT
females...	23	14	16	22	19	11	15	10
males.....	22	23	17	22	15	12	9	8
Total	45	37	33	44	34	23	24	18

The per cents of recombinations were as follows:

	<i>per cent</i>
Black-pink. 54.0
black-bent.... 45.5
pink-bent..... 52.0

Here we cannot obtain the per cents of recombinations between sex and the other factors, since the parent which was heterozygous for the other factors was not the one which was heterozygous for sex.

CONCLUSIONS

The foregoing experiments prove that the gene for bent wings segregates independently of the sex-linked group of genes and of the two hitherto known non-sex-linked groups; accordingly, the genes of *Drosophila* now fall into four divisions, one sex-linked, corresponding to the X-chromosome, and three non-sex-linked, corresponding to the three pairs of autosomes. Thus the chief gap yet remaining in the series of genetic phenomena that form a parallel to the known cytological facts in *Drosophila ampelophila* has now been filled. It may therefore be predicted that no genes undergoing independent assortment of those at present known can hereafter be discovered in individuals of *Drosophila ampelophila* that show the chromosome configuration normal to the species, and it also seems probable that when other mutations are discovered in the fourth group, the genes in which they occur will be found to be linked strongly to the gene for bent wings, since

the fourth chromosome is probably the small one, and so any genes in it must lie near together. The close parallel existing between the number and relative *sizes* of the groups of genes and of the chromosomes leaves little doubt that it must be the pair of small chromosomes with which the factor for bent wings is connected, and that mutations occur more frequently in larger groups of genes, which are connected with larger chromosomes, than in smaller groups; mutation therefore would happen pretty much at random, in that it would usually take place about as often in one group as in another of equal size.⁵ This, too, makes it probable that the mutations in the larger chromosomes have occurred at various points scattered throughout their whole length, and are not confined, *as a group*, to a particular region or regions. The exceptional case of no mutations having been observed in the Y-chromosome, as we have seen, does not really form an argument against this view, which other facts support. That more mutations have been found in the X-chromosome than in either of the two long autosomes, which are nevertheless about the same size as X, is also to be expected, because a larger proportion of the mutations occurring in X would be noticed, since the male flies manifest all genes present in their single X, whereas in the case of other chromosomes, any mutant gene that is recessive to normal cannot manifest itself unless it be present in duplex. Granting, then, the correspondence between size and number of chromosomes and of groups of genes, it is difficult to see why larger groups of genes should follow the distribution of the larger chromosomes unless we conceive the connection between the genes and the chromosomes to be that the genes are material particles actually lying in and forming a part of the chromosomes with which they go. In any case, we must admit that the occurrence of a mutation in a fourth independent group of genes in *Drosophila* forms a further argument, if any more still be needed, in favor of the chromosome theory of heredity.

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⁵ This should not be taken to mean that any particular gene mutates as often as any other; it is definitely known that, both in *Drosophila* and in other forms (corn. *Marabilis*, etc.), some genes are more likely to mutate than others.

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THE EFFECTS OF CENTRIFUGING THE SPERMATOCYTE CELLS OF NOTONECTA, WITH SPECIAL REFERENCE TO THE MITOCHONDRIA

ETHEL NICHOLSON BROWNE

SIX FIGURES

The spermatocyte cells of *Notonecta* show a very definite distribution of substances, including the mitochondria, when subjected to a centrifugal force. The method of procedure was to dissect out the testes from the insects, put them together with a little Ringer's solution in small glass tubes and centrifuge them for two or three hours with a water centrifuge. The testes were then either teased out on a slide and the cells stained with an intra-vitam stain, Janus green, and examined, or were fixed in Flemming's fluid, sectioned and stained with iron hemotoxylin.

The normal resting spermatocyte cells of *Notonecta* have been described in a former paper (Browne '13). The cytoplasm contains scattered mitochondria of two sorts, fibers and spheres, and the nucleus contains a deeply staining karyosphere in which the chromatin material is collected (fig. 1). When a cell of this sort is centrifuged, the mitochondria are driven to the distal¹ pole, where they appear as a deeply staining mass in which individual bodies cannot be distinguished except along the edge (fig. 2). The mitochondria are therefore the heaviest material in the cell. The rest of the cell is filled with clear cytoplasm. The nucleus goes to the centripetal¹ pole, showing that it is of less specific gravity than the cytoplasm. The karyosphere is always driven to the distal end of the nucleus, and in some cases in fact, is driven through the nuclear membrane.

¹ The term 'distal' is used in this paper to denote the pole away from the axis of the centrifuge, the term 'centripetal' to denote the pole nearest the axis of the centrifuge.

In younger spermatocyte cells, as I have described, there is besides the scattered mitochondria, a flat plate of mitochondrial material lying just outside the nuclear membrane, and a mass of mitochondria projecting from it into the cytoplasm. When cells of this type are centrifuged, this plate and mass of mitochondria remain in position, the rest of the mitochondria are driven to the distal pole (fig. 3). In these young cells, there is an aggregation of oil drops at the extreme centripetal pole; these can be distinguished only in the living material, probably being dissolved in the process of fixing and staining. The nature of these drops has been determined by means of Soudan III. The oil drops are not present in the older spermatocyte cells, having apparently been used up during the growth of the cells.

In the dividing cells, the mitochondria are likewise thrown to the distal pole by the centrifuge. The spindles and asters are, in many cases at least, perfectly normal and are not interfered with by the shifting of mitochondria, but lie in approximately the normal position in the clear protoplasm. Since it has not been determined how long one of these cells normally takes to divide, it is not possible to say whether the chromosomes actually pass to the poles while the cells are in the centrifuge, but various anaphase stages have been found in the centrifuged cells showing that the chromosomes are undergoing normal division. The spindle axis may lie in any direction with reference to the mitochondrial mass. In figure 4, the spindle axis is approximately parallel to the mitochondrial mass; in figure 5, approximately perpendicular. Figure 6 shows a spindle in polar view and its relation to the mass of mitochondria. In some cases, a few of the mitochondria apparently get caught by the spindle or asters and thus appear on the centripetal side of the division figure (figs. 5 and 6).

When the cells are centrifuged and then left in Ringer's solution for several hours or even overnight, the materials do not become redistributed over the cell, but they remain in the position induced by the centrifuge.

It is of interest to compare the centrifuged spermatocyte cells with centrifuged egg cells. In a number of marine eggs, it has

been found by Lyon ('07), Morgan ('10) and Lillie ('08) that there are at least three layers of materials segregated out by the centrifuge. There is at the centripetal pole a gray cap, then a layer of clear protoplasm, and at the distal pole a mass of yolk and pigment. This is true in the eggs of *Arbacia*, *Asterias*, *Nereis*, *Chaetopterus*, *Phascolosoma*, *Cerebratulus* and *Cumingia*. The gray cap, in some cases at least (*Nereis*, *Chaetopterus*, *Cumingia*) is formed of oil drops and corresponds therefore, in nature and in position to the cap of oil drops in the centrifuged spermatocyte cells. The middle layer of clear protoplasm is practically the same in the two cases. The heaviest material in the egg cells is the yolk and pigment, and this therefore corresponds with the mitochondria in the spermatocyte cells, but whether any of the mitochondria have the same function as the yolk spheres of the marine egg is questionable. In a very recent paper, Beckwith ('14) has shown that there are also three layers in the centrifuged eggs of *Hydractinia*, and she has been able to distinguish between yolk and mitochondria, which are mingled together at the distal pole. This is the first attempt, so far as I know, to segregate the mitochondria from the protoplasm by experimental methods, and it is interesting to find that her results for the egg tally with my own for the spermatocyte cells.

In comparing the unfertilised marine egg and the resting spermatocyte cell of *Notonecta* the resemblance is very striking. The normal cells in the two cases show a close similarity in appearance, and react in the same way to the centrifuge. In the case of the egg, the germinal vesicle is forced toward the centripetal pole by the centrifuge, and the nucleolus to the distal end of the nucleus. The nucleus and the karyosphere in the spermatocyte cells take the same relative positions. In the dividing cells of the marine eggs, it has been found in general that the mitotic figure remains intact when the cells are centrifuged, just as it does in the dividing spermatocyte cells. The whole figure may be shifted in the eggs so as to lie in the clear protoplasm. This does not occur to any considerable extent in the spermatocyte cells, though there might be a very slight shifting in some cases.

The main conclusions that can be drawn from these experiments are: (1) Three layers of materials can be segregated in the spermatocyte cells of *Notonecta* by means of the centrifuge, a cap of oil drops, a clear protoplasmic layer and a layer of mitochondria. (2) The mitochondria are definite bodies which can be separated from the protoplasm proper, and they are the heaviest material in the cells. (3) Normal division figures occur in spite of the redistribution of materials.

Marine Biological Laboratory.

Woods Hole, Mass.

July 7, 1914.

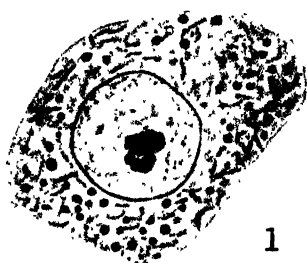
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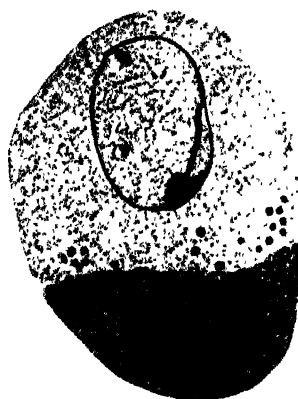
PLATE 1

EXPLANATION OF FIGURES

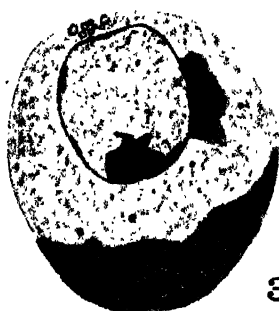
- 1 Normal resting spermatocyte cell, showing distribution of mitochondria.
- 2 Older resting spermatocyte cell, centrifuged. The gray mass consists of mitochondria.
- 3 Younger resting spermatocyte cell, centrifuged, showing oil drops, and mass of mitochondria against nuclear membrane.
- 4 Dividing cell, centrifuged; late anaphase.
- 5 Dividing cell, centrifuged; early anaphase.
- 6 Dividing cell, centrifuged; polar view of metaphase.



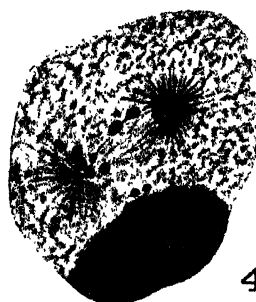
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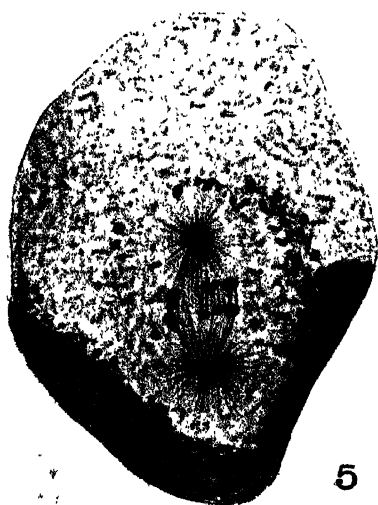
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FERTILITY AND STERILITY IN DROSOPHILA AMPELOPHILA

III. EFFECTS OF CROSSING ON FERTILITY IN DROSOPHILA¹

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ONE DIAGRAM

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¹ This part of the work was carried out at the Marine Biological Laboratory at Woods Hole, Mass. The writer is indebted not only to the management of the Station, but also to the Zoological Department of Columbia University for placing at his disposal every opportunity for carrying forward the present study. I wish to express my appreciation to Prof. T. H. Morgan for the unfailing interest he has taken in these studies.

INTRODUCTION

This paper, which is the third of the series on "Fertility and Sterility in *Drosophila ampelophila*," considers the evidence that bears on the effects of crossing on fertility when: (1) Stocks from the same source (descended from the same grandparents) separately inbred are crossed; (2) Stocks from different sources separately inbred are crossed; (3) When the mutations, having pink eyes and white eyes are crossed with each other and with a wild stock which has been inbred (brothers and sisters) for 26 generations.

The evidence so far presented goes to show that some of these stocks have, up to the time the present experiments were carried out, lost in fertility on inbreeding. The evidence, however, does not show conclusively that the loss is due to the process of inbreeding as such. The fact remains, that the different strains did up to this time lose in fertility on continuous inbreeding, and it will now be shown what effect out-crossing has on these reduced strains. It is to be recalled that 'fertility' is here used in the sense of eggs that reach maturity.

There are some experimental data that bear out the belief that the crossing of different races or strains is beneficial to the *fertility of the stock itself*. This relation has been demonstrated beyond any doubt in the second part of this paper in so far as it applies to the truncate mutant for the fertility of the female was more than doubled when mated to a wild stock, while the fertilizing powers of the truncate male were more than three times as great with a wild female as compared to his own mate.

I wish now to consider the evidence that bears on the question as to whether or not a rise in fertility will result when one strain of *Drosophila* is crossed with any other strain. The evidence deals especially with the fertility of two mutations and two wild stocks that originally came from two different sources. It will facilitate treatment of this subject to give a brief résumé of the history of these stocks.

HISTORY OF THE STOCKS USED IN CROSSING

1. *The Woods Hole stock*

This is a wild stock that originally came from Woods Hole, Massachusetts. How long it had been in captivity I do not know, but when I took charge of the stock it showed a fertility of 77.3 per cent. The stock was tested at various times throughout the year 1912-1913. The behavior of the fertility of this stock is given in table 1. The stock was cultured *en masse*.

TABLE 1

Showing the fertility of the Woods Hole stock

TIME TESTED	NO. EGGS ISOLATED	NO. FLIES HATCHED	PER CENT FERTILITY
September 11 to September —, 1912	1402	1084	77 3
September 16 to October —, 1912	1622	1227	75 0
January 20 to February 14, 1913	2085	1421	70.0
June 28 to July 15, 1913	917	581	63 4
July 15 to July 28, 1913	531	317	59.7
August 7 to August 24, 1913	601	431	71.7
August 17 to September 4, 1913	856	715	83.6
October 21 to November 11, 1913	467	341	73.0

2. *The inbred stock*

This stock originally came from Falmouth, Massachusetts. During the course of the experiments brothers and sisters have been selected and paired for over 26 generations. Its productivity was at the beginning of the experiments relatively high as shown by the large number of offspring produced. On inbreeding the stock gradually dropped in productivity as shown in table 2.

TABLE 2

Showing the productivity of the inbred stock in successive generations of close inbreeding

GENERATION	1	2	3	4	5	6	7	8	9	10	11	12	13	14
No. offspring produced		368	209	190	184	65	119							159

This evidence taken by itself does not prove conclusively that the loss in productivity was due to a decrease in the fertilizing power of the gametes. That this is a very probable interpretation however is shown by the fact that in the F_{14} generation when the productivity dropped to 159 per pair their fertility was only 32 per cent. And yet the flies of the F_{14} generation were producing twice as many fertile sperm and twice as many fertile eggs as shown by outcrossing into the truncate stock (Part II, diagram E).

3. I_2 and I_3 stocks

In the fifth generation of the inbred stock, when they were producing 184 offspring to the fertile pair, I set aside two different bottles besides a number of pairs that were kept to continue the inbred stock. All of these flies had descended from the same grandparents, a single pair of the third generation. In one bottle there were three females and four males. This I shall designate as I_2 . In the other bottle there were ten males and twenty-two females. This I shall refer to as I_3 . These flies were set apart April 2, 1912, and received no attention except that the flies were transferred to clean bottles about every three weeks and fresh food added from time to time. It is important to bear in mind that we have three stocks descended from the same germ-plasm.

4. *The white-eyed stock*

This stock arose as a mutation from the inbred stock early in the history of the strain. This stock received the same treatment as I_2 and I_3 .

5. *The pink-eyed stock*

This is an eye mutation that arose in one of Morgan's cultures.² It had been bred for some three years. I received my stock from Mr. Liff, a graduate student in the department, who has made a study of their productivity. This stock was bred in mass culture for about four months when the present experiments were carried out.

² Science, vol. 33.

We have then six different strains, two from different localities and some that had the same germ plasm. I now propose to examine the fertility of the different stocks and their behavior on crossing. The combinations were made up as shown in diagram A.

METHODS

In order to get an exact measure of fertility the eggs were isolated as described in Part II. From the stock bottle in each case 15 virgin females and 30 young males were selected. The flies that hatched from June 19 to June 26 were separated every twelve hours from the stock bottles. There can be no question as to the virginity of the females. The males and females were kept in separate bottles and the different combinations made up on June 26. An epidemic of mating took place in all the bottles a short time after the flies came out from under the influence of the ether.³ I commenced to isolate the eggs two days later. This process was carried out in the same way as in previous experiments with one exception. The weather was very warm and the larvae emerged from the egg in less than twenty-four hours. In order to exclude this source of error I added another bit of food after isolating the eggs. This served as food for the parents. After six or eight hours this food was removed and a new bit added from which the eggs were later isolated. Tables 3a, 3b, and 3c give the number of eggs isolated each day and the corresponding number that hatched.

DISCUSSION

A study of diagram A brings out the essential relations that concern the questions propounded at the beginning of this paper. 1. What is the effect on fertility when germ plasm originally from the same source separately inbred for several generations is recombined by crossing? It will be recalled that I_1 , I_2 and I_3 represent in this case the stocks under study. It is to be noted that the three stocks although originally brothers and sisters and descended from

³ Mr. A. H. Sturtevant has shown that the mating habit in this species is largely associated with the sense of smell and this fact probably accounts for the phenomenon here observed.

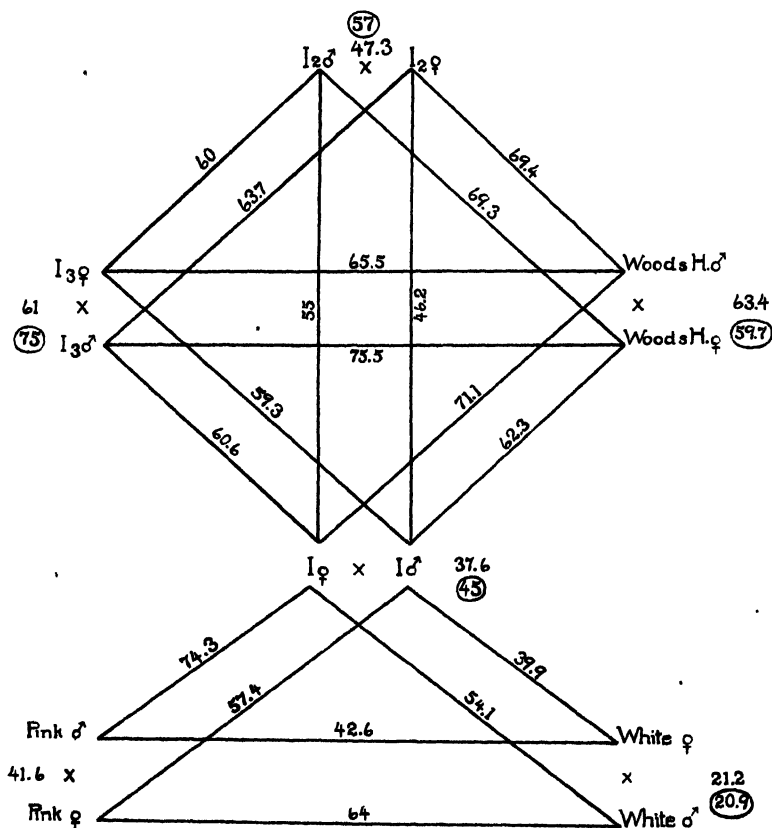


Diagram A Based on the foregoing data, showing different combinations and the fertility in each case.

the same grandparents, show different degrees of fertility. The inbred stock in which brothers and sisters had been continually selected had lost most in fertility. In the crosses it will be observed that there is no sudden rise in fertility as one might expect but, on the other hand, a peculiar relation exists in that the stock in each case having the highest fertility is able on crossing to bring the fertility of the lower stock up to its level and this is true whether through the male or through the female.

TABLE 3 a

Showing the number of eggs isolated day by day and the corresponding number that hatched from each of the combinations. The first letter in each combination represents the stock from which the female was taken, the second letter the stock from which the male was taken. T = truncate; I = inbred, I₂ = second inbred stock from 9 males, 4 females; I₃ = third inbred stock from 10 males, 22 females; WH = Woods Hole stock; P = pink-eye stock; W = white-eye stock

DATE	1913	1 (CONTROL) T × T		2 I ₂ × I ₂		3 I × I ₂		4 (CONTROL) I ₂ × I ₂		5 I ₂ × I ₃		6 (CONTROL) I ₂ × I ₂		7 I ₂ × WH	
		Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults
June 28		0	0	48	27	37	21	80	39	60	25	40	23	25	21
29		36	7	21	17	34	24	35	24	47	27	61	29	56	42
30		56	14	50	32	29	20	55	32	60	31	36	16	0	0
July 1		50	13	30	19	40	26	40	29	48	25	60	26	38	31
2		37	4	25	12	40	17	25	7	32	21	71	18	20	6
3		48	2	40	18	33	20	33	14	0	0	49	25	22	9
4		45	7	30	14	65	26	22	11	60	26	50	23	26	13
5		52	6	56	27	57	39	73	41	52	21	85	20	30	33
6		42	10	64	36	36	16	50	14	26	7	75	31	50	34
7		50	11	75	51	50	29	67	55	75	61	55	35	55?	41
8		20	9	33	23	28	24	17	15	36	20	24	10	12	10
9		22	5	28	21	24	12	30	25	42	26	44	17	0	0
10		43	4	75	30	60	26	35	24	50	26	75	40	25	12
11		60	13	40	24	0	0	50	35	60	25	60	37	60	46
12		30	8	50	39	75	60	21	19	75	44	75	38	37	24
13		9	5	25	16	40	22	20	16	55	34	65	41	13	6
14		0	0	30	26	33	25	0	0	35	30	40	26	17	4
15		0	0	20	11	20	9	0	0	15	8	20	11	16	7
Total		600	118	740	443	701	416	653	400	828	457	985	466	522	339
Per cent		20		60		59.3		61		55		47.3		61.5	

TABLE 3b

⁸ (control) WH × WH		⁹ I ₁ × WH		¹⁰ I × WH		¹¹ I × I ₁		¹² I ₁ × I ₁		¹³ (control) I × I		¹⁴ I ₁ × I		¹⁵ WH × I ₁		¹⁶ WH × I	
Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults
36	20	47	30	35	25	22	11	25	11	19	4	38	20	53	46	35	21
18	11	31	20	60	45	24	20	21	11	18	13	42	21	42	30	65	33
40	24	27	16	50	26	45	25	38	28	71	37	25	12	62	57	62	51
28	19	35	22	45	23	42	22	0	0	26	8	60	33	48	38	40	36
18	7	20	7	25	13	30	16	35	25	30	13	40	24	50	27	19	13
15	10	61	42	45	29	28	13	41	19	17	6	29	13	100	50	31	20
55	28	8	4	25	19	35	17	50	26	31	7	45	15	50	30	65	23
51	28	43	25	63	53	68	27	90	37	60	15	64	27	100	63	35	15
80	47	50	29	46	28	30	18	67	50	40	10	75	31	52	50	40	13
80	60	41	33	75	65	80	57	66	45	40	18	50	31	100	80	90	67
100	69	23	20	32	21	30	21	12	9	45	18	38	21	64	56	45	38
60	46	35	32	51	38	30	16	31	22	22	6	23	8	57	47	40	33
76	38	0	0	64	34	50	26	35	20	50	17	65	25	72	45	75	32
60	30	60	44	60	44	40	22	40	25	50	14	60	20	60	30	60	20
55	35	70	61	100	84	80	64	75	63	60	25	50	35	68	64	70	49
63	53	40	29	40	26	20	11	60	50	43	17	39	24	75	70	55	40
42	32	45	33	40	35	35	26	34	21	15	11	30	12	40	39	50	44
40	24	30	15	40	29	40	30	20	9	20	7	30	0	40	33	30	17
917	581	666	462	896	637	729	442	740	471	657	246	805	372	1133	855	907	565
63.4		69.4		71.1		60.6		63.7		37.6		46.2		75.5		62.3	

TABLE 3c

17 W H X I ₁		18 W X I		19 I X P		20 P X I		21 (control) P X P		22 W X P		23 (control) W X W		24 I X W		25 P X W	
Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults
45	30	41	22	0	0	10	6	36	22	50	38	28	11	10	5	60	46
53	44	40	18	0	0	0	0	30	9	16	4	20	4	14	10	38	26
60	48	18	9	32?	11	10	5	35	23	15	5	33	8	31	23	80	63
50	33	36	14	20	14	21	17	32	17	26	16	25	10	28	13	61	38
35	21	22	8	14	10	26	16	34	7	24	4	7	4	29	17	42	15
49?	26	31	11	7	5	20	10	56	15	7	2	42	8	22	13	47	24
82	42	55	15	10	3	55	28	20	4	43	12	25	1	17	2	30	7
58	38	20	7	47	31	80	53	75	28	14	2	40	3	85	50	85	38
48	31	35	6	19	16	18	6	50	15	17	8	10	0	21	9	41	19
90	76	27	9	80	60	40	23	40	19	40	24	50	9	23	11	60	47
75	66	8	0	37	34	0	0	7	5	10	5	40	7	34	16	31	21
54	41	15	3	26	19	17	13	24	17	13	7	50	13	27	11	50	43
65	30	21	7	60	44	43	20	46	10	35	8	29	2	60	31	81	50
50	10	60	20	60	46	70	31	60	17	50	21	65	13	70	30	70	35
65	59	50	32	70	60	85	54	80	44	45	23	55	19	60	41	40	40
50	41	43	25	30	24	60	29	20	12	22	10	65	12	45	17	50	39
30	28	33	21	40	36	40	30	50	24	42	18	35	7	27	19	26	17
20	14	35	7	20	12	20	12	40	18	40	10	30	7	10	4	18	15
979	678	590	234	572	425	615	353	735	306	509	217	649	138	613	332	910	583
69.3	-	38.7	-	74.3	-	57.4	-	41.6	-	42.6	-	21.2	-	54.1	-	64	-

The second question under consideration is; What is the effect on fertility when stocks from different sources separately inbred are crossed? The relations expressed by the right hand side of diagram A throw some light upon this question. In two of the combinations high fertility brought the low fertility up to its level while in four of the combinations there is an appreciable rise in fertility beyond that shown by the parents, although by no means as high as one might expect from the history of the crosses made with the truncates in Part II. But since the rise in fertility occurs here in the combinations expressed by the right side of the diagram and not those on the left it looks as if the rise in fertility in this case is significant. It must be admitted, however, that the rise in fertility in these crosses is not great enough to base a final conclusion upon, in regard to a point as far-reaching as this. It is to be remembered that Woods Hole, Massachusetts, is only four miles distant from Falmouth and it is altogether probable that the two strains had not been separated by many generations when taken into captivity. Consequently, this material would present after all a picture very much like the first case. If the environment can influence the different strains in respect to the 'factors' that bring about fertility the influence in this case has been slight, and, after all, from stocks so closely related this is probably what we should expect to find. In any case it is certain that the high-producing stocks can bring the fertility of the low-producing stocks to their level whether descended from the same or from different germ plasms. It might seem from this (although I am far from contending at present that such is necessarily the case) that there is a set of factors of some sort for fertility and that when a loss in fertility occurs in a stock (other than that which occurs in the case of mutations) it is the same set of factors that is lost or is changed and this accounts for the fact that there is no rise in fertility on crossing. The stock that has the highest fertility (the largest number of factors) acts as a dominant character and brings the lower set of factors up to its level. In the case of a sudden rise in fertility on crossing such as occurs in the case of some of the mutations it is probable that the stocks have lost different factors for

fertility, and that crossing gives the proper constellation of factors for a marked rise in fertility.

It must be frankly admitted that this explanation at best is only tentative and that the door must be left open for further investigation. The possibility of transmissible lethal factors is not to be overlooked.

The third question under consideration relates to the effect on fertility of crossing certain mutations, namely, pink-eye and white-eye and the effect on fertility when these are crossed into the inbred stock. The triangular portion of diagram A shows that there is a rise in fertility in all cases. In four of the six cases the rise in fertility is very high. This is analogous to what happens in the case of the truncates when crossed into other stocks.

I wish here to add the data from an experiment that has some bearing on the foregoing considerations and also some bearing on the question of inbreeding. The object of this experiment is to serve as a check upon the controls used in the foregoing experiments, namely, Woods Hole, white-eye, I_2 , I_3 and the inbred stocks. This experiment was carried out in the same manner as the foregoing. The egg counts began the same day that the former experiment closed, July 15, 1913. The number of eggs isolated, together with the corresponding number that hatched, is given in table 4.

I have placed the percentage of fertility of these stocks in diagram A and enclosed the numbers in circles. It will be noted that the Woods Hole and white-eyed stocks remain practically the same as in the previous experiment. There is a marked rise in fertility, however, in case of the three stocks originally from the same germ plasm. Later (Aug. 7 to 24, table 1) the Woods Hole stock was tested. Its fertility had risen from 59.7 to 71.7. The Woods Hole stock was again tested August 17 to September 4. This stock now gave a fertility of 83.6. The fact is that all my stocks at this time showed a marked rise in fertility, as is evidenced by the experiments to be dealt with in the next paper. Even the truncate stock which had been tested many times through the year and had varied from 20 to 26 per cent now gave a fertility

of 31 per cent. Table 1 shows that in the case of the Woods Hole stock the fertility gradually fell throughout the year. I fully expected to find that the fertility would be continually reduced on inbreeding, and yet the fertility of the stock rose in August and September until it was actually higher than it was during the September of the previous year by 6.3 per cent. The meaning of this is obscure. It will be recalled that Castle found seasonal fluctuations in productivity in his flies. The period, however, in which low productiveness prevailed in his stocks corresponds in a general way with the period of high fertility in my stocks.

In the light of this evidence it would seem that inbreeding as such cannot be the *vera causa* of the low fertility that usually accompanies the process. It would seem from this evidence and the evidence presented by Moenkhaus that the fertility of a stock could be maintained, and the closest inbreeding practiced, provided

TABLE 4

Showing the fertility of the Woods Hole, white-eyed, inbred, I₁ and I₂ stocks. 1A = Woods Hole stock; 2A = white-eyed stock; 3A = I₁ stock; 4A = I₂ stock; 5A = inbred stock, F₃₅ generation

DATE 1913	1A		2A		3A		4A		5A	
	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults
July 15	95	65	75	21	70	58	53	33	75	32
16	50	34	33	4	40	31	35	21	67	31
17	45	30	50	15	40	35	50	40	30	14
18	44	31	30	8	50	42	60	41	32	15
19	29	14	51	20	45	34	50	26	50	26
20	30	10	50	11	66	40	45	22	30	11
21	31	15	31	2	40	33	25	7	35	16
22	41	24	25	3	35	30	35	14	12	6
23	21	11	31	4	25	19	40	22	20	6
24	30	21	26	3	17	13	40	26	16	9
25	25	12	25	3	17	6	22	8	22	12
26	50	29	25	5	32	27	22	16	33	18
27	25	13	11	2	37	26	25	14	20	10
28	15	8	35	5	30	18	25	12	25	6
Total.....	531	317	498	106	544	412	527	302	467	212
Per cent.....	59.7		20.9		75.7		57.3		45.4	

the proper combinations were made. It seems probable in the case of fertility, as in many other characters, that inbreeding gives a chance for defects to be brought to the surface; and that low fertility is likely to accompany close inbreeding provided it is not guarded by rigorous selection. When a stock has reached a low degree of fertility it seems strange that that same stock should be able to rise again in fertility. Yet this is exactly what may happen. Take for example the truncate fly which has been selected for 75 generations and has its fertility reduced to about 20 per cent; and yet that fly can throw a form, the long wings, the fertility of which is more than twice as great as its truncate brothers and sisters.

The fact that different individuals, brothers and sisters of the same stock, should differ in such a marked degree (so that one is actually able to separate the more fertile ones from the less fertile flies by inspection) is submitted as evidence to show how selection may operate in controlling the fertility in these strains.

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FERTILITY AND STERILITY IN DROSOPHILA AMPELOPHILA

IV. EFFECTS ON FERTILITY OF CROSSING WITHIN AND WITHOUT AN INCONSTANT STOCK OF DROSOPHILA

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ELEVEN DIAGRAMS

INTRODUCTION

In Part II of these studies it was shown that although the mutant stock truncate, produced a large number of fertile sperm and fertile eggs, yet when the truncate female was mated to the truncate male only 20 per cent of the eggs hatched. It was also shown that the truncates were not a homogeneous stock, for the flies with truncate wings give rise to offspring some of which have long wings like those of the wild flies. This has held true through many generations of continuous selection. These long winged flies in turn are also not homogeneous because they throw some truncates, and this has held despite some twenty (estimated) generations of selection.¹ A peculiar phenomenon shown by the long wings is that their fertility when tested together is about twice as great as that shown by their truncate brothers and sisters when tested together.

I wish here to present in detail the evidence that bears on the result of crossing within this inconstant stock; and the effects on fertility when both forms are crossed into a wild stock—the Woods

¹ Since both forms are under study in this paper it is convenient to refer to this stock as an "inconstant stock."

Hole stock. I shall also consider the behavior of the fertility of the extracted truncates (extracted after crossing out to a wild stock) when tested together and when back-crossed into their low-producing and high-producing grandparents.

FERTILITY OF THE EXTRACTED TRUNCATES

I wish to deal first with the evidence that bears on the question raised in Part II, as to whether or not the fertility of the truncate stock can be raised by outcrossing. In other words is low fertility in this case a concomitant of the truncate wing condition, or can high fertility be transferred to the truncate stock by crossing it out to a wild stock and extracting?

To answer this question a truncate female was crossed to a Woods Hole male. This cross I shall refer to as A. The reciprocal cross in which the truncate male was mated to the Woods Hole female I shall refer to as B. There were five bottles of the P_1 made up in each case. A large number of the F_1 were mated in pairs and the truncates selected from their offspring.

The fertility of the extracted truncates was then tested by means of the following combinations:

1. A truncate female (F_2 extracted from the truncate grandmother) was mated with four of her truncate brothers.

2. A truncate female (F_2 extracted from the truncate grandfather) was mated with four of her truncate brothers.

3. Control: The fertility of the original truncates was tested by placing a number of males with a single female in each case.

The results of this test are recorded in tables 1, 2 and 3. The fertility of the extracted truncates when tested together is almost 50 per cent, while the fertility of the truncates used for control is only 22.6 per cent. In other words, from a source of fertility of 63.4 per cent (Woods Hole stock; Part III, table 3b, no. 8) there has been transferred to the truncate winged forms (originally with a fertility of about 20 per cent) about 25 per cent of additional fertility. The increase in fertility is the same in this case whether the truncate male or female is used as the grandparent.

TABLE 1

Showing the result of testing the fertility of the $A\varphi \times A\sigma^7$ (F_2 truncates extracted from truncate grandmother)

NO.	TOTAL NO. EGGS LAID	NO. EGGS ISOLATED	NO. FLIES EMERGED	AGE OF ♀ WHEN COUNT BEGAN	LIFE OF ♀ IN DAYS	AVERAGE NO. EGGS LAID PER DAY	PER CENT OF EGGS WHICH COMPLETE DEVELOPMENT
11	861	353	128	5	47	25 0	33 4
12	211	184	80	5	30	8 5	43.4
13	17	17	2	5	18		11.8
15	513	328	180	5	31	19.8	54.8
16	575	370	182	5	35	19 2	49 2
17	403	250	118	5	33	14.4	47.2
24	356	292	134	4	17	27 4	45 8
25	610	392	210	4	32	21 8	53 5
26	445	310	176	4	25	21 2	56 7
27	552	370	198	4	29	22 1	53 6
28	413	331	168	4	24	20 7	50 8
29	791	441	210	4	41	21 4	47 6
Total.....		3638	1786				

Per cent of eggs which complete development 49.1.

TABLE 2

Showing the result of testing the fertility of the $B\varphi \times B\sigma^7$ (F_2 truncates extracted from truncate grandfather)

NO.	TOTAL NO. EGGS LAID	NO. EGGS ISOLATED	NO. FLIES EMERGED	AGE OF ♀ WHEN COUNT BEGAN	LIFE OF ♀	AVERAGE NO. EGGS PER DAY	PER CENT OF EGGS WHICH COMPLETE DEVELOPMENT
16	581	370	182	5	35	19.4	49 2
20	*	1	1	5	25		
21	297	231	78	5	27	13.5	33.7
22	352	279	158	5	25	17.6	56.6
23	67	42	13	5	22	4 0	31.0
30	657	463	242	4	29	26.3	52.3
32	266	266	116	2	23	12.6	43.6
Total.....		1652	790				

Per cent of eggs which complete development 47.8.

*Laid eggs which went to pieces.

TABLE 3

Control: Showing the result of testing the grandparental stock, truncate by truncate

NO.	TOTAL NO. EGGS LAID	NO. EGGS ISOLATED	NO. FLIES EMERGED	AGE OF ♀ WHEN COUNT BEGAN	LIFE OF ♀	AVERAGE NO. EGGS PER DAY	PER CENT OF EGGS WHICH COMPLETE DEVELOP- MENT
1	154	26.	4	7	28	7.3	15.4
3	62	62	16	7	16	7.0	25.8
4	287	265	73	7	26	15.1	27.5
5	227	227	43	7	35	8.1	18.9
7	36	36	10	6	14	4.0	27.8
8	258	238	54	6	27	12.3	22.7
10	226	126	22	6	41	6.4	17.5
Total		980	222				

Per cent of eggs which complete development 22.6.

It is true that the extracted truncates vary somewhat in the length of wing. Moreover, they appear in about the ratio of 1 truncate wing to 14 of the long wings. In all cases I selected the most typically truncate forms, and if low fertility is an accompaniment of the truncate wing as such, I should have discovered the fact in this experiment. This does not exclude the possibility, however, that some of the low fertility here may be concomitant to the truncate wing, for a comparison of the fertility of their long winged brothers and sisters shows that the fertility of the extracted longs is higher (see table 7).

In Part II it has been demonstrated by numerous experiments that the truncate stock is deficient in egg production. A comparison of the egg production of the extracted truncates together with that of the truncates used in control and also with that of the truncates given in Part II makes it absolutely certain that these extracted truncates have been benefited also in regard to egg productivity as a result of crossing and extracting from a wild stock. The evidence is conclusive that fertility can be transferred to the truncates.

PRODUCTIVITY OF THE EXTRACTED TRUNCATES

If it be true that the fertility of the extracted truncates is more than twice as great as the original truncates and if it be true that the egg production of the extracted truncates is increased, it follows that if the extracted truncates be bred together in pairs their productivity should be greatly increased. To judge from the previous history of the truncates in regard to productivity (Part II; table 1) and the attendant low fertility we should expect that by doubling the fertility of the gametes of the truncates that they would produce between 100 and 125 offspring on an average to the fertile pair instead of 50 as formerly. The number of offspring produced above 125 would, in a rough way, give a measure of the increased egg production. This does not take into consideration, any difference that may exist in the length of life between the truncates and extracted truncates. It would seem from the tables that the extracted truncates had been benefited to some slight degree in respect to the length of life as a result of extracting from a wild stock.

In order to throw some light on the question just raised, I bred together a number of these extracted truncates in pairs. These flies were from the same source as those used in the previous experiments. The pairs were made up August 9 and 10 and discontinued on September 1. The final count was made September 11; consequently the total number of children produced is not represented. The results are given in tables 4a and 4b. Ten pairs of truncates were used to control this experiment but it was evident from inspection that their productivity was practically the same as in former experiments and consequently the offspring were not counted. A study of the table makes it certain that it is possible not only to put fertility into the truncate stock but also increased egg production. Consequently the productivity of the truncates has been raised from 50 to about 300 as a result of crossing out and extracting. Since the fertility of the extracted truncates is twice as great and the productivity six times as great it follows that egg production has been increased three fold—from 200 to

about 600. A comparison of tables 1 and 2 where the egg production of the extracted truncates is given with table 3 and the many tables in Part II where the total output of eggs of the original truncates is represented will show that the above conclusion is warranted.

TABLE 4a

Showing the result of breeding together the extracted truncates. $A \text{♀} \times A \text{♂}$ (F_2 truncates extracted from truncate grandmother)

NO.	TOTAL NO. OFFSPRING
1	336
2	295
3	408
4	330
6	364
7	no offspring
8	299
14	250
15	271
16	265
17	211
18	228

TABLE 4b

$B \text{♀} \times B \text{♂}$ (F_2 truncates extracted from truncate grandfather)

NO.	TOTAL NO. OFFSPRING
5	203
9	196
10	390
11	162
12	363
13	127
19	no offspring
20	123

FERTILITY OF THE EXTRACTED TRUNCATES BACK-CROSSED TO THEIR GRANDPARENTS, THE ORIGINAL TRUNCATE STOCK AND THE WOODS HOLE STOCK

The foregoing pages have demonstrated the marked difference in fertility that exists between truncates and extracted truncates. I wish here to consider the effects on fertility when the extracted truncates are back-crossed to their low-producing grandparents (truncates) and their high-producing grandparents (Woods Hole). I shall consider first the evidence that bears on the effect on fertility of back-crossing extracted truncates with truncates. This experiment was carried out at the same time as experiment I in this paper. The facts are recorded in table 5.

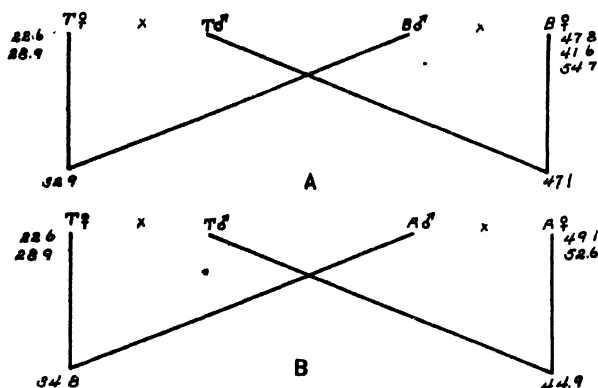


Diagram A Expresses the effect on fertility of crossing the extracted truncates B (in which the Truncate male was used as the grandfather) with the original truncates, T.

Diagram B Expresses the effect on fertility of crossing the extracted truncates A (in which the truncate female was used as the grandmother) with the original truncates, T.

The flies of table 5 were mated July 21 and the egg count began July 25. The sexes were kept separate for about four days before mating. The results of these crosses are brought together for comparison in diagrams A and B.

TABLE 5

Showing the effect on fertility of back-crossing the extracted truncates with the original truncate stock. T = truncate, all from family No. 178. A = truncate extracted from truncate grandmother. B = truncate extracted from truncate grandfather

NUMBER	COMBINATION	NO. OF FEMALES	NO. OF MALES	NO. EGGS ISO-LATED	NO. FLIES EMERGED	PER CENT OF FERTILITY	TRUNCATES	LONGS
33	B ♀ × T ♂ No. 178.....	10	20	938	442	47.1	102	117
34	A ♀ × T ♂ No. 178.....	8	20	811	365	44.9	149	88
35	B ♂ × T ♀ No. 178.....	10	22	613	201	32.9	41	72
36	A ♂ × T ♀ No. 178.....	11	21	247	86	34.8	21	15
37	Control B ♀ × B ♂.....	7	15	637	265	41.6	64	105
38	Control A ♀ × A ♂.....	7	18	754	396	52.6	148	147
39	Control T ♀ No. 178 × T ♂ No. 178	15	34	668	194	28.9		
40	Control B ♀ × B ♂.....	12	21	931	510	54.7	80	120

In table 5 I have added as a matter of record the number of long-wings and short-wings that appeared in a number of bottles from which complete counts were made. No definite ratios can be given for comparison with the study of wing ratios made in Part II, where it was shown that the truncates threw 1 long to 7 = truncates, the longs in turn threw 1 truncate to 7 = longs. The ratio of the longs to the truncates in the crosses made here is near equality.

The foregoing experiments bear on the effects on fertility when the extracted truncates are back-crossed with their low-producing grandparents. I wish next to consider the effects on fertility when the extracted truncates are back-crossed with their high-producing grandparents—the Woods Hole stock. The experiment was carried out in the usual way. The results are given in table 6.

The relations brought out in this experiment are expressed in diagrams C, D, and E.

Diagrams A and B show the result on fertility of back-crossing the extracted truncates with the low-producing grandparental truncates. It is to be noted first of all that the controls show the fertility of the extracted truncates to be practically twice that of the original truncates. Moreover, the fertility of the extracted truncates is the same whether the truncate male or female is used as the grandparent. On crossing, there is no great rise in fertility beyond that shown by the parents, as has been demonstrated in the case of the truncates crossed to wild races. The extracted truncate female is able to bring the fertility of the truncate male up to its level but not beyond. The extracted truncate male, on the other hand, does not bring the fertility of the truncate female up to its level by about 15 per cent. It is to be noted that the fertility shown in diagram A is practically the same as in diagram B, indicating that here the effect is the same on the truncate grandchildren, whether descended from the truncate male or truncate-female.

In contrast with the conditions found in these crosses, we find, that when the extracted truncates are back-crossed to the high-producing parents there is a sudden and distinct rise in fertility; beyond that of either parent stock (diagrams C and D). The

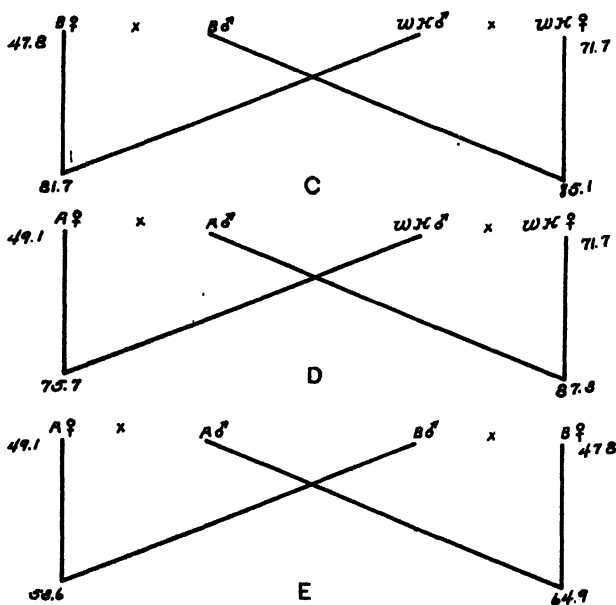


Diagram C Showing the effect on fertility of crossing the extracted truncates B (F_2 truncates descended from the truncate ♂) with the high-producing grand-parental stock.

Diagram D Showing the effect on fertility of crossing the extracted truncates A (F_2 truncates descended from the truncate ♀) with the high-producing grand-parental stock.

Diagram E Showing the effects on fertility of crossing the extracted truncates A (F_2 truncates descended from the truncate ♀) with the extracted truncates B (F_2 truncates descended from truncate ♂).

behavior of the fertility of the extracted truncates toward the Woods Hole stock is practically the same whether the truncate flies descended from the truncate grandmother or truncate grandfather.

It is to be emphasized that the fertility of the truncates has been practically doubled (as determined by testing brother and sister) as a result of crossing out and extracting; and yet a marked degree of incompatibility exists between the gametes of the extracted truncates. For example, diagram C shows that the extracted male B produced 85.1 good sperm and the extracted female B produced 81.7 good eggs and yet the fertility as shown by testing

TABLE 6

Showing the effect on fertility of back-crossing the extracted truncates with their high-producing grandparents—the Woods Hole stock. WH = Woods Hole. B = extracted truncate (F_2 extracted from truncate male); A = extracted truncate (F_2 extracted from truncate female); M = new wild stock captured at Woods Hole, Mass., used for control.

DATE 1913	WH σ^7 × B σ^9		WH σ^7 × A σ^9		WH σ^9 × A σ^7		WH σ^9 × B σ^7		WH σ^9 × WH σ^7		A σ^7 × B σ^9		A σ^9 × B σ^7		M σ^9 × M σ^7 (CONTROL)	
	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults
Aug. 7.....	40	36	44	5	20	17	43	33	20	12	70	33	41	18	51	51
8.....	50	38	31	18	40	30	40	36	45	40	50	19	30	15	50	46
9.....	51	43	60	42	50	43	45	39	37	26	50	38	50	26	60	60
10.....	61	38	60	43	20	17	50	43	41	36	70	40	50	26	70	67
11.....	50	33	76	66	60	52	60	50	56	47	65	50	52	29	66	61
12.....	45	41	16	14	70	70	50	44	40	28	50	38	14	9		
13.....	54	49	45	42	50	41	53	45	28	17	32	25	13	10		
14.....	36	33	37	31	60	52	65	56	32	24	30	18	53	21		
15.....	40	32	42	35	50	45	45	39	37	21	32	21	35	20		
16.....	30	27	40	34	31	27	22	18	28	17	32	23	28	19		
17.....	25	22	40	23	36	30	30	27	40	30	35	24	19	13		
18.....	15	12	30	25	35	30	26	24	23	16	22	19	11	6		
19.....	15	14	30	23	17	16	20	17	20	16	17	14	11	9		
20.....	14	13	19	14	15	13	30	25	22	16	15	10	6	4		
21.....	11	9	20	16	8	8	16	13	3	3	17	13	21	13		
22.....	20	15	30	25	21	18	30	27	22	18	40	25	18	11		
23.....	25	23	30	27	59	54	30	24	56	34	35	22	36	22		
24.....	35	26	60	53	66	55	70	57	51	30	30	17	30	17		
Total.....	617	504	710	536	708	618	725	617	601	431	692	449	518	278	297	285
Per cent of fertility..	81.7		75.5		87.3		85.1		71.7		64.9		53.6		96	

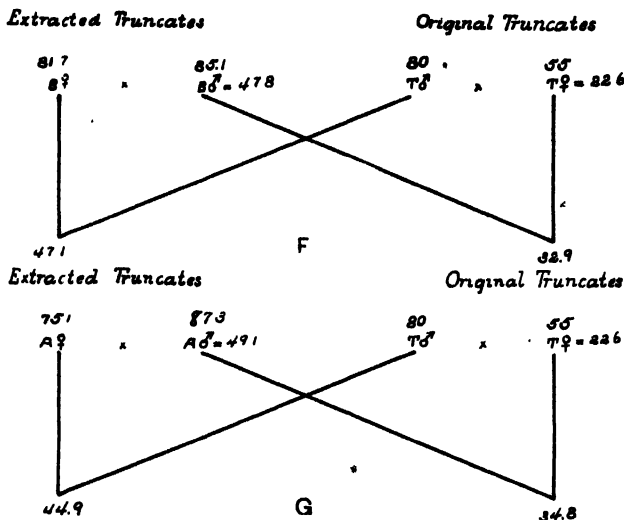


Diagram F Showing incompatibility that exists between truncates and extracted truncates (extracted from truncate ♂).

Diagram G Showing incompatibility that exists between truncates and extracted truncates (extracted from truncate ♂).

together was only 47.8. Diagram D shows practically the same condition.

As just stated the extracted truncates produce 81.7 good eggs and 85.1 good sperm (diagram C). We know from many former experiments that the truncates produce about 80 per cent of good sperm and 55 per cent of good eggs. Yet when these gametes are brought together, as shown in diagrams A and B there is a marked degree of incompatibility. Diagrams F and G based on this and former experiments, will show at a glance the facts that relate to incompatibility in this case.

It remains to be pointed out that there is a rise in fertility beyond that shown by either parent when the truncates extracted from the truncate grandfather B are crossed with the truncates extracted from the truncate grandmother A (diagram E). It seems strange that there should result a rise in fertility in this cross and also when the extracted truncates are back-crossed into their high-producing grandparental stock (diagrams C and D); and yet there results no rise in fertility when the extracted truncate female is back-

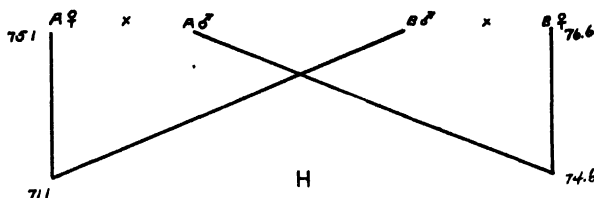


Diagram H Showing the fertility of the extracted longs when mated together and when crossed with each other.

crossed to the truncate male; and that in the reciprocal cross there is shown not only no rise in fertility but an actual incompatibility (diagrams A and B).

It has been shown that the fertility of the truncates is raised from about 25 per cent to 50 per cent by crossing out to a wild stock and extracting. It must be remembered, that the fertility of the wild stock from which the truncates were extracted was far below 100 per cent. It seems altogether probable that had a more fertile stock been used the fertility of the extracted truncates would have been still higher.

FERTILITY OF THE LONG-WINGED BROTHERS AND SISTERS OF THE EXTRACTED TRUNCATES

The question still remains as to how the long-winged brothers and sisters of the extracted truncates behave in respect to fertility when tested together. The data recorded shown in table 7 gives an answer to the question.

The results of this experiment as expressed in diagram H show that the long-winged brothers and sisters of the extracted truncates are more fertile than the extracted truncates (in this case by over 25 per cent). It would seem from this that while it is possible to put a certain amount of fertility into the gametes of the truncates, yet a certain degree of incompatibility remains with the truncate wing as evidenced by the fact that their long-winged brothers and sisters give a higher degree of fertility. It is to be noticed in this case that there is no rise in fertility on crossing. The fertility in the crosses is practically the same as in the controls.

EFFECT ON FERTILITY OF CROSSING THE TRUNCATES WITH THEIR LONG-WINGED BROTHERS AND SISTERS AND THE EFFECT ON FERTILITY WHEN BOTH OF THESE FORMS ARE CROSSED INTO THE WOODS HOLE STOCK

I wish to return to a consideration of the low fertility of the truncate stock and consider more especially the difference in fertility between the truncates and their long winged brothers and sisters. I shall also consider the behavior of fertility when the truncates are crossed to their long winged brothers and sisters, and also the behavior of fertility when both forms of the inconstant stock are out crossed to the Woods Hole stock. Table 8 shows the combinations made up, the number of eggs

TABLE 7

Showing the fertility of the long-winged brothers and sisters of the extracted truncates when tested together and when crossed with each other. A = F_2 long-wing extracted from truncate grandmother. B = F_2 long-wing extracted from truncate grandfather

DATE 1913	A ♀ × A ♂		B ♀ × B ♂		A ♀ × B ♂		B ♀ × A ♂	
	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults
August 15	30	21	40	26	65	51	50	31
16	31	23	30	21	45	33	50	42
17	50	35	35	19	40	24	40	23
18	30	17	36	30	27	18	41	35
19	20	13	44	33	22	18	29	22
20	23	18	19	15	29	17	30	22
21	13	7	40	33	25	20	31	15
22	49	36	27	23	60	41	45	37
23	40	37	70	58	41	28	57	42
24	80	60	71	59	60	55	60	48
25	50	37	63	50	50	39	40	29
26	50	35	86	73	70	50	53	47
27	40	38	52	35	55	35	45	37
28	50	34	60	39	50	37	50	38
29	40	32	40	39	50	25	51	37
30	47	40	16	13	40	24	25	16
Total	643	483	739	566	729	515	697	520
Per cent of fertility	75.1		76.6		71.1		74.6	

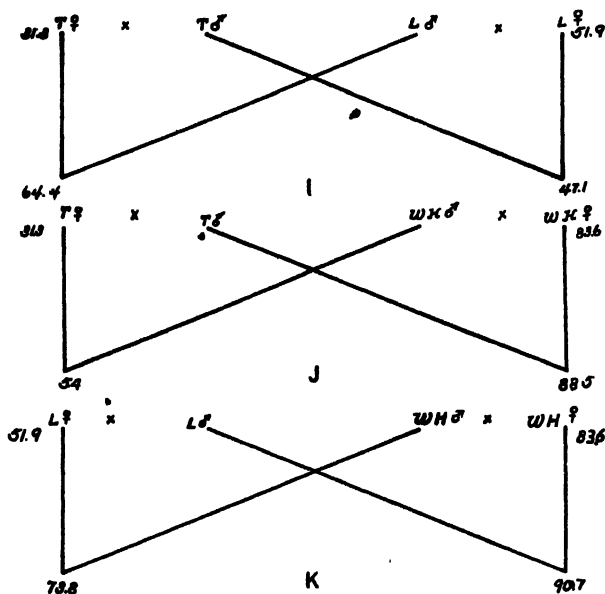
isolated day by day and the corresponding number of eggs that hatched.

The truncates in this experiment gave a fertility of 31.3 which is from 5 to 10 per cent higher than in any of the previous experiments. The Woods Hole stock shows a fertility of 83.6 which is much higher than that of previous experiments. The fact is that all the stocks at this time showed a rise in fertility. Whatever the meaning of this may be, the former relations hold as to the effect on fertility when crossed, as is evidenced by the fact that there is a marked rise in fertility when the truncate is crossed into the Woods Hole stock; compare diagram J with diagram C, Part II.

Diagram I brings out the effect of crossing within the truncate stock. The fertility of the truncates when tested together is 31.3 while that of their long-winged brothers and sisters when tested together is 51.9. When the longs are crossed to the truncates as the diagram shows, the high fertility of the longs is able to bring the fertility of the truncates up to its level and it would seem that in the case of the cross between the truncate female and the long male that there was a marked rise in fertility. The fact is that in this experiment this cross is higher by 10 per cent than in the case where the truncate female is tested with a wild male. I have made many crosses between the truncate female and the Woods Hole male with the result that the fertility of the combination stands almost invariably at 55 per cent. It would seem from this experiment and the evidence from former ones that the rise in fertility of this particular combination is significant.

A study of the diagrams will show a marked degree of incompatibility to exist between the flies with the long wings for while 51.9 of their eggs hatch when mated together yet no less than 90.7 of the sperm and 73.8 of the eggs are capable of entering into a combination that results in development, as is shown by crossing into the Woods Hole stock (diagram K).

The greatest degree of incompatibility however exists between the flies with the truncate wings for in this experiment they produced no less than 54 per cent of good eggs and 88.5 per cent of



Diagrams I, J and K Showing the effects on fertility of crossing within and without the inconstant truncate stock. T = truncate. L = long-wing thrown by truncates. WH = Woods Hole.

Diagram I Crosses between truncates and their long-winged brothers and sisters.

Diagram J Crosses between truncates and the Woods Hole stock.

Diagram K Crosses between the long-winged flies thrown by the truncates and the Woods Hole stock.

good sperm and yet only 31.3 of the eggs from the truncate females hatched when mated to the truncate males (diagram J).

It remains to be pointed out that a certain degree of incompatibility exists in the cross made between the truncate male and his long-winged sister. For their fertility when tested together is 47.1 and yet he is producing 88.5 fertile sperm and she is producing 73.8 fertile eggs as shown by outbreeding into the Woods Hole stock. In the cross between the truncate female and the long-winged male the incompatibility seems to be removed. For while the long-winged male is producing 90.7 fertile sperm the female is producing only 54 per cent of fertile eggs. Here, as I have

TABLE 8

Showing the effect on fertility of crossing the truncates with their long-winged brothers and sisters and the effect on fertility when both of these forms are crossed into the Woods Hole stock. *T* = truncate. *L* = long-wings from truncates. *WH* = Woods Hole stock

DATE 1913	¹ <i>T</i> ♂ × <i>T</i> ♀ (CONTROL)		² <i>T</i> ♂ × <i>L</i> ♀		³ <i>L</i> ♂ × <i>T</i> ♀		⁴ <i>L</i> ♀ × <i>L</i> ♂ (CONTROL)		⁵ <i>T</i> ♂ × <i>WH</i> ♀		⁶ <i>T</i> ♀ × <i>WH</i> ♂		⁷ <i>L</i> ♀ × <i>WH</i> ♂		⁸ <i>L</i> ♂ × <i>WH</i> ♀		⁹ <i>WH</i> ♀ × <i>WH</i> ♂ (CONTROL)	
	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults
Aug. 17	34	5	22	14					55	45	35	15	21	17			32	26
18	25	4	31	11	15	3	14	4	40	30	17	3	45	29	27	21	25	25
19	10	3	27	8	18	7	13	2	30	30	17	8	35	11	56	48	23	19
20	10	1	38	11	13	5	23	9	41	40	15	7	25	13	30	39	25	20
21	23	9	26	14	15	8	25	9	42	33	10	6	30	20	40	33	5	3
22	40	12	50	26	50	30	31	13	50	47	18	9	33	22	100	92	53	42
23	47	22	60	27	92	56	30	12	100	88	30	18	50	46	100	97	78	60
24	64	22	90	52	70	36	60	19	100	88	40	32	60	48	52	51	85	66
25	50	23	41	12	63	40	30	12	50	46	4	3	51	44	51	42	53	40
					55	37												
	56	23			52	34			61	57							60	51
26	50	17	53	26	43	30	35	20	53	45	5	2	50	43	50	42	52	42
27	40	14	34	27	40	33	42	23	60	56	5	2	17	12	40	40	50	43
28	50	19	50	19	60	30	40	24	25	24	6	4	33	26	40	40	50	40
29	57	10	47	27	45	27	37	50	40	32			8	7	44	36	50	44
30	32	11	43	18	49	29	30	22	40	36			5	4	46	44	40	35
31	30	10	30	16	28	16	34	24	40	34					25	20	40	33
Sept. 1	40	13	35	19	37	27	40	31	28	24					30	30	39	31
2	35	7	30	18	30	15	30	13	43	38					24	23	41	37
3	25	5	27	14	26	15	13	9	50	47					20	14	40	40
4	35	6	20	6	28	14	16	16	31	26					40	37	20	18
Total	735	236	754	365	829	534	543	282	979	866	202	109	469	346	815	739	856	715
Per cent of fertility	31	3	47.1		64.4		51.9		88.5		54		73	8	90.7		83.6	

pointed out, the incompatibility is removed and we have a distinct rise in fertility.

This evidence would seem to indicate that a marked difference in respect to fertility may exist between brothers and sisters of the same stock. In this case we find a great degree of incompatibility between the flies with a definite wing form—long-wings; the greatest degree of incompatibility between flies with truncate wings. We also find a marked degree of incompatibility when the truncate male is crossed to his long-winged sister but no incompatibility in the reciprocal cross.

ON THE EARLY PULSATIONS OF THE POSTERIOR LYMPH HEARTS IN CHICK EMBRYOS: THEIR RELATION TO THE BODY MOVEMENTS

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From the Anatomical Department of the Johns Hopkins University

TWO CHARTS

INTRODUCTION

The lymph hearts of the chick were discovered by Budge in 1882. Previous to this, pulsating lymph hearts had been described in adult aquatic birds and in the ostrich and cassowary, by Stannius and Panizza. Budge found that lymph hearts, although absent in adult chickens, are present in the embryo and function from the ninth day to the time of hatching. He was able to inject these lymph hearts, from the lymphatic vessels surrounding the allantoic arteries, in embryos of ten days and over, and he describes them as two rounded sacs, situated on each side, at the angle between the tail and pelvis, and superficial to the myotomes. He studied the muscles in the wall of the lymph heart by dissection of fixed preparations. Budge also discovered the lymph heart in living chicks, of eight days and over, and found that its pulsations did not coincide with those of the blood heart. Budge concluded that, since the lymph hearts make their appearance at approximately the same time as the lymphatic vessels of the allantois and atrophy with the disappearance of the allantois, they are intimately connected with the function of that organ.

A more extensive study of the lymph hearts of the chick was published in 1900 by Sala. The lymph hearts, according to Sala, in embryos of eight days, are clear almond-shaped sacs situated on either side of the tail, lateral to the myotomes and somewhat ventral to the caudal margin of the os ilii. After the

tenth day, the hearts grow more rounded in shape and become surrounded by a layer of fat. With the gradual reduction of the curve of the vertebral column, they eventually occupy a more dorsal and anterior position with reference to the os ilii. Sala found that the lymph hearts communicate with the intersegmental coccygeal veins—at first connecting with the first five of these veins, and, in later stages, with three of them.

From his study of the lymph heart in cross sections, Sala found that, in embryos of seven and one-half days, its walls are made up of mesenchyme which penetrates the lumen of the sac in the form of trabeculae. In nine-day chicks, muscle fibers begin to differentiate in this connective tissue, and eventually they form a layer in direct contact with the endothelial lining. In embryos of thirteen days the muscle fibers and the endothelium are separated by a layer of mesenchyme.

Sala also investigated the earlier stages of the lymph heart, in cross sections of chicks six and one-half to seven days old. He found a row of irregular spaces connected with the first five intersegmental coccygeal veins and concluded that the lymph heart was formed by the flowing together of these spaces.

Mierzejewski in 1909 studied the superficial lymphatics of chick embryos by injection. He confirmed the results of Sala as to the development of the lymph heart but states that the anlage of the lymph heart first appears in chicks of five and one-half days, instead of six and one-half days, as Sala had stated. Mierzejewski agrees with Sala as to the time of appearance of the muscle fibers in the wall of the lymph heart and states that, at nine days, the lymph heart begins to pulsate and beats once to every eight or ten contractions of the blood heart.

The authors have studied this region in the stages of embryos investigated by Sala and Mierzejewski and also in earlier stages. We have found that the spaces in the mesenchyme, described by Sala as the anlage of the lymph heart, are in reality definite enlarged lymphatic capillaries, which form a continuous plexus connected with the intersegmental coccygeal veins, and whose endothelial lining can be demonstrated easily by injection with dilute silver nitrate, and also by the microscopic study of sections.

At a much earlier stage—in chicks of four and one-half to five days—lymphatic vessels are present, although their size is much smaller, and in embryos of five to six days, the lymphatic plexus, which precedes the definite pulsating lymph heart, normally contains stagnant blood, which enters it through its connections with the intersegmental veins of the tail. Preliminary reports of studies made of this lymphatic plexus in living embryos and by the method of injection, have been published. A more complete account of these researches, together with careful microscopic studies of the endothelium is soon to be published.

In the course of this study of the developing lymph heart, we noticed, while examining a chick of seven days under the binocular microscope, that there occurred several beats of the lymph heart simultaneously with vigorous movements of the tail. As muscular contractions of body, tail and limbs had previously been seen in embryos of various ages, there was suggested a study of the early pulsation of the lymph heart in relation to these movements.

For these studies we used a binocular microscope, enclosed in a warm chamber which was heated by an electric light bulb or a small alcohol lamp. An opening was made in the shell and the shell membrane over the region of the embryo, was carefully removed. Throughout the observation, the embryo was kept moist by the occasional addition of a few drops of Ringer's solution, warmed to 38 to 39°C. When the eggs were opened in this way, the chicks remained alive and healthy for from five to eight hours.

THE MUSCULAR MOVEMENTS OF CHICK EMBRYOS

When an egg, incubated four days or more, is opened in this manner the embryo is seen to be undergoing violent movements. These movements are found to belong to two types: one in which the embryo as a whole is moved, with a continuous back and forth swaying motion around the umbilical cord as a center, and the other type, in which the movements are spasmodic and affect various parts of the embryo. The first type is caused by the peristaltic contractions of the amnion, described by von Baer in 1828.

Von Baer observed movements in chicks of five days which he believed to be due to the exposure of the embryo to the cold air. On the seventh day he states that the movements have become more general: "Der Embryo schwingt in Amnion hin und her, auf dem Nabel, wie auf einem befestigten Stiele—Es schien mir daher eine Art unregelmässiger Pulsation im Amnion." Von Baer found that the contractions of the amnion were very active in chicks of eight or nine days and that after that time they became weaker.

The subject of amniotic contraction was further investigated by Remak in 1854. After opening the egg, he noticed violent wave-like movements of the amnion, which were now and then followed by quieter rhythmical contractions. When the contractions ceased they could be started again by pricking the amnion with a needle. Pieces of amnion, examined under a simple lens, were found to contract when stimulated. Remak found smooth muscle fibers in the amnion, which increased in abundance up to the tenth day of incubation; after this time their number diminished. Nerves, according to Remak, are not present in the amnion. Like von Baer, Remak doubted the presence of these peristaltic contractions in normal embryos inside the egg, and stated that the violent contractions were probably caused by exposure to the air.

This last point was refuted by Vulpian in 1857, who observed the embryo in unopened eggs held before a bright light. He saw the swaying movement of the embryo in chicks of five to eight days and found it to be violent and continuous in the unopened eggs, under normal conditions. He also described contraction of the under surface of the allantois in chicks of twelve days and over.

Preyer in 1885, studied these contractions of the amnion, as well as the other type of movements in chick embryos. He studied chicks on each day of their development, by means of a specially constructed ooscope, both before and after opening the shell and found that the amniotic contractions first appeared in embryos of four days, and continued up to the thirteenth day of incubation. At this time, the violent swinging of the

embryo becomes a gentle swaying and, in later stages, all movement is absent. Preyer showed that the amniotic contractions, contrary to the ideas of von Baer and Remak, were not caused by the cold air, but were normally present in warm eggs and ceased a short time after exposure of the egg to cold air.

Preyer's method enabled him to keep the embryos alive and active for only a short time after opening the shell. By making use of the warm chamber and the binocular microscope, we have been able to watch the passive and active movements of the chick for several hours after opening the shell, and to study them in greater detail. We found that the amniotic contractions follow each other in rapid succession: each peristaltic wave consumes on an average, 3.3 seconds, and about 4.5 seconds elapses between the cessation of one wave and the beginning of the next one. We also observed, in a five-day chick, four distinct types of amniotic contraction: (1) The peristalsis starts at the top of the head and passes completely down to the tail of the embryo; (2) The contraction starts at the same place but extends only half as far, i.e., to the posterior edge of the wing; (3) The wave starts over the neck bend, half way between the ear vesicle and the anterior edge of the wing and extends simultaneously in both directions; (4) The contraction wave starts posteriorly and extends from the tail to the head end of the embryo. Types (1) and (4) were found to occur most frequently, and in type (1) the passive movement of the whole embryo was the greatest. With this type there is evidently a rapid circulation of the amniotic fluid for as the contraction wave passes posteriorly, the fluid is squeezed out and the amnion comes in contact with the embryo, and the amount of fluid increases in the relaxed part of the sac. The extremities are pressed tightly against the body wall, as the amniotic wave passes over them. This undoubtedly has an effect on the form of the chick, for in embryos which had lived for several hours with the amnion removed, we noticed that the extremities, on the side away from the yolk, showed a tendency to grow away from the body until they often projected at right angles to it.

The other type of movements—the active contractions of the embryo—have also been recorded by many observers. Preyer, in an extensive review of the older literature on the subject, mentions that such observations were made by Harvey in 1651, by Béguelin in the middle of the eighteenth century, and by Home in 1822, as well as by von Baer and Remak. Most of these observers, however, did not distinguish between the passive movements of the embryo, caused by the contraction of the amnion, and the active movements of the chick itself. However, Dareste, in 1879, noticed movements in an embryo without an amnion, and thus demonstrated conclusively the independence of this second type of movement.

Preyer gives an interesting and comprehensive account of these active movements in chick embryos. From the first to the fifth day of incubation he found no embryonic movements, aside from the beating of the heart. Mechanical and electrical stimulation of the embryo produced no effect. On the fifth day, Preyer first observed the active embryonic movements and the amniotic contractions. The first movements of the embryo, according to Preyer, are simple in character and consist chiefly in a bending of the back from side to side. The head, tail, and extremities are moved passively at this stage. In successive later stages, the movements become more numerous, violent and complicated, as the various parts of the embryo differentiate. At the sixth and seventh day, the bending of the body becomes more pronounced, the tail contracts independently, the head nods, and the paddle-like extremities are moved inward and outward, and from the eighth to the eleventh day, the movements continue to increase in strength and variety. Intervals occur at all these stages, during which the embryo does not move. During this second period of development—from the fifth to the eleventh day—Preyer found no response of the musculature to mechanical stimulation or to injury, such as amputation of a leg. However he occasionally observed a weak response of certain muscles, following electrical stimulation of the spinal cord, on the ninth and tenth day of incubation.

In the third stage of development described by Preyer—the period from the eleventh to the fifteenth day—the active movements of the embryo continue to occur, at frequent intervals, and, in addition to those present in younger chicks, movements of the individual toes and opening and closing of the eyelids and beak occur frequently. At this stage, reaction to mechanical stimulation is doubtful: at times a seemingly definite response is obtained and, at other times, the embryo remains motionless when stimulated, although continuing to move spontaneously. At this stage, however, chicks responded definitely to electrical stimulation, by movements of the limbs, toes, beak, etc., according to the point at which the stimulus was applied.

In the last period of development—from the sixteenth to the twentieth day of incubation, Preyer states that spontaneous movements are much less frequent, but that the chicks respond readily to electric stimulation by tetanic contraction, and show an increased tendency to respond reflexly to mechanical stimulation.

With his especially constructed ooscope, Preyer was able to observe, at all stages, both types of embryonic movements, those due to the contractions of the amnion, and those due to contractions of the muscles of the embryo itself, in the unopened egg, kept at incubator temperature, thus proving that they are normal, and not caused by outside factors introduced at the time of opening the shell.

Our observations of the muscular movements of chicks were confined to embryos of four to fourteen days, since this comprises the important periods in the development and early functioning of the lymph heart. Our observations agree with Preyer's description of the character of these movements, and of their first appearance in chicks of four days. However, in addition to the movements of the various parts of the embryo, described by Preyer, we noticed that sudden violent jerks, which seemed to involve the entire musculature of the embryo, occurred at all of the stages studied. With regard to the absence of all reaction to mechanical stimulation, in chicks between the fifth and eleventh day, our results are in entire agreement with those of Preyer.

One very striking characteristic of the active movements of chicks during the period of their development from four to fourteen days, is their periodicity. The movements occur in definite groups or spasms followed by an intermission or period of rest, after which the movements recur. This periodicity was not noted by Preyer or by earlier observers, although they record the fact that the active movements were not continuous.

While the active movements of the embryo can be observed with the amnion intact and have been studied through this membrane, they are more easily observed when it is cut across and drawn aside. In observing the periodic movements, we kept records of the minute and second at which each spasm commenced, its duration, and the interval from the beginning of one spasm to the beginning of the next. We found that the duration of the periodic movements and the periods of rest varied in embryos of different ages. For example, in a chick of six and one-half days, the duration of the periodic spasms averaged 13 seconds and the period of rest 39 seconds, while in an embryo of nine days, the movements were found to continue for 28.5 seconds and to be followed by an average period of 54.5 seconds, in which the chick did not move. In all the stages studied, one or two movements of the tail; a slight twitch of the leg or wing, or a single jerk of the whole body occasionally occurred, apart from the periodic spasms. The interval from such an isolated movement until the beginning of the next was always shorter than the interval between the true periodic spasms.

We also made some observations of the reaction of embryos to changes in temperature. When the temperature of the warm chamber was raised until a small thermometer placed near the embryo registered 41° , the active movements ceased, after a few minutes, and the chick remained motionless until the temperature was lowered to 38 or 39° , when the periodic movements were resumed. We also found, as Dareste had observed in the case of a chick without an amnion, that the muscular movements ceased when the temperature was lowered to 35° or under and began again when the temperature was raised. When the amnion was left intact, the response of the body movements to

a change of temperature was delayed. In this case, when the temperature was raised to 41° or lowered to 35°, the peristaltic contractions ceased and the amnion relaxed, allowing the embryo to sink deep into the yolk. The periodic movements persisted for several minutes, in such instances, probably owing to the more sheltered position of the embryo.

The movements of fish and amphibian embryos in the egg have been studied by many investigators. Preyer reviewed the literature on the subject and published many new observations on the embryos of the trout and the frog. He finds both active and passive movements occurring in these embryos: the passive movements are due to the almost continuous rotation of the embryo inside the egg, caused by the action of the cilia. The active movements, according to Preyer's description, resemble greatly those present in chick embryos of four to five days, the body bends from side to side and the head and tail bend at the same time. More recently, Harrison, Hooker, Wintrebert and Coghill have observed movements in early amphibian embryos, and Paton has studied them in embryo fish. No periodic movements were noted by any of the investigators of amphibian material. Mention is made of spontaneous movements, but as a casual and not a frequent occurrence. Harrison distinguishes two types of response to stimulation in young amphibian embryos: (1) the non-nervous type, characterized by a sharp tonic contraction and (2) the nervous type which resembles a swimming movement. The former has been shown to be the more primitive, since it is noticed in embryos before the peripheral nerves have developed (Wintrebert) and has also been demonstrated in embryos in which the spinal cord had been cut or removed (Harrison and Hooker). It appears, then, that the early movements of chick embryos differ from those noted in amphibia, by occurring periodically and with greater frequency, and also in the fact that they can not be instigated or altered by mechanical stimuli, such as pressure or direct piercing of the muscles with glass needles.

The early movements of fish embryos, described by Paton show a closer resemblance to the movements of chicks. In

Selachian embryos of 3.5 to 4 mm., according to Paton, jerky lateral movements begin. These are rhythmic in character: several movements occur, followed by a pause of several seconds and then the movements begin again. They do not appear to be affected by mechanical stimulation of the embryo, although they are increased by squeezing the yolk sac. The rhythm of the movements is altered by heat, by brilliant light, and by interfering with the normal swing of the head from side to side. In all of these cases, the movements become more violent. In the case of approaching death, the contractions become tonic. Fish embryos appear to resemble chicks in the periodic character of their early movements and in their failure to respond to mechanical stimuli.

In Mammalia, Preyer (pp. 586-595) studied the movements in guinea-pigs. The earliest movements were seen in a 'fourth week' embryo of 16 mm. which "bewegte den Rumpf in situ stark." In this embryo, he notes "Zehen noch nicht getrennt." In all embryos of successive weeks, movements were seen, increasing in intensity. In the 16 mm. embryo electrical stimulation over the spinal cord caused muscle contractions, and in 26.4 mm. embryos peripheral stimulation, both electrical and mechanical, led to reflex movements.

That periodic muscular movements, beginning at least as early as the fifth day of incubation and continuing with increasing intensity throughout the remainder of incubation, take place in developing chicks (and that movements occur in mammalian embryos as well, beginning at least as early as 16 mm. in guinea pigs—Preyer) is a fact of much significance in the study of the physiology of the embryo and of the mechanical factors concerned in development, especially of muscles, bones and joints—a fact which has been frequently overlooked.

Thus Thoma, in his study of bone growth, takes up the mechanical factors acting on cartilage at the time of beginning ossification. On page 350 he says: "Als Ursache der Belastung kann demgemäss im wesentlichen nur die Spannung der Hautdecken und der Muskulatur." No mention is made of the tension caused by contraction of the muscles.

Again R. Schmidt, p. 95, in discussing the ossification of bones in embryos, concludes "Da die Grundzüge der Architektur sich ausbilden, ehe noch die Muskelemente kontraktile sind (Henneke, Bernay, u. a.) kann die Ursache nicht unmittelbar in Gebrauch zu suchen sein." Herbst (p. 7), after quoting the above, adds "so schliesst Schmidt ganz richtig." The tension produced by muscle contractions, left out of

account by these observers, must be quite considerable, since, according to our observations, chicks of six days move very actively, approximately one quarter of the time, a total of six hours out of the twenty-four, chicks of nine days, one-third of the time or eight hours out of the twenty-four, while up to the thirteenth or fourteenth days the time of the movements increases still further.

Harrison, on the other hand, has made a significant beginning at studying the relation between muscular movement and the development of muscles and bones. He found that muscles differentiate relatively normally in tadpoles whose movements are prevented by the use of chloretone, as well as by the removal of nerves. He also observed that ossification occurred in the hind limb of a tadpole into which no nerve had grown, and hence in which there was no muscular movement.

In a new-born calf E. H. Weber found total absence of the spinal cord and nerves below the first thoracic vertebra. Corresponding to the absence of nerves, the muscles were entirely absent, in the posterior part of the body, and while the bones of the hind limb were present and described as 'normally developed' yet the hind limbs weighed only one-half as much as the fore limbs, no description is given of their architecture, and all the joints were ankylosed. Between these two sets of observations there is a wide gap in which it remains to be determined *to how great an extent* the muscular movements in embryos regulate the development of the typical architecture of bones, the form of joint surfaces and the growth of muscles.

THE PULSATION OF THE LYMPH HEART

In our observations of the pulsation of the lymph heart, in chicks of various stages, we kept careful records of the exact time at which each spasm of muscular movements commenced, the length of the interval between movements, and the number of lymph heart beats occurring during the observation.

As already stated, in chicks of 5 to 6 days, the region in the angle of the pelvis and tail, later occupied by the lymph heart contains a plexus of lymphatic capillaries filled with stagnant blood. At this stage, the movement of the tail from side to side, at the time of each periodic spasm of body movements, is violent enough to cause a blanching of this whole region, particularly noticeable in the superficial blood capillaries. But the definite localized contractions so characteristic of the beating lymph heart of later stages, are not yet present.

Stage 1. In embryos of 6 to 7 days (measuring from 19 to 22 mm. greatest length, before fixation) the stagnant blood is

no longer present in the plexus of the posterior lymph heart region. In the living chick, this area has a swollen, translucent appearance and, upon injection, a plexus shows up, which is more compact and which is composed of fewer and larger vessels than the lymphatic plexus of younger embryos. At this stage, distinct localized pulsations of this clear area are noticeable in living chicks. Physiologically, then, a posterior lymph heart is now present.

Embryos of this stage were kept alive and under continuous observation for from 2 to 5 hours and records made of every lymph heart contraction and of each periodic spasm of body movements. It was found that the lymph heart beats occurred invariably at the time of the periodic spasms and never in the intervals between these body movements. Moreover we found that, at this stage, a beat of the lymph heart was always accompanied by a movement of the tail. Tail movements occurred, unaccompanied by any lymph heart contraction, but beats of the lymph heart unaccompanied by a tail movement were never observed.

After an embryo of this stage had been observed for an hour and this relation of lymph heart beats to the periodic body movements recorded, chlorotone (1 : 4000 in Ringer's solution at the temperature of the chick) was dropped on the embryo. A minute later the body contractions ceased and the chick remained motionless for a period of fifteen minutes. During this time no pulsation of the lymph heart was observed. As the effect of the anaesthesia wore off the spasmodic contractions returned and soon regained their former periodicity. The lymph heart beats returned at the same time and accompanied the body spasms as before and each beat was always associated with a contraction of the tail. It is evident, then, that at this earliest stage in which definite pulsations of the posterior lymph heart are visible, the beating is inseparably connected with the periodic movements of the embryo (charts 1 and 2: Stage 1).

We found, however, that unlike the rest of the musculature of the chick, the lymph heart muscle responds to direct stimulation and always contracts when it is punctured. In chicks of

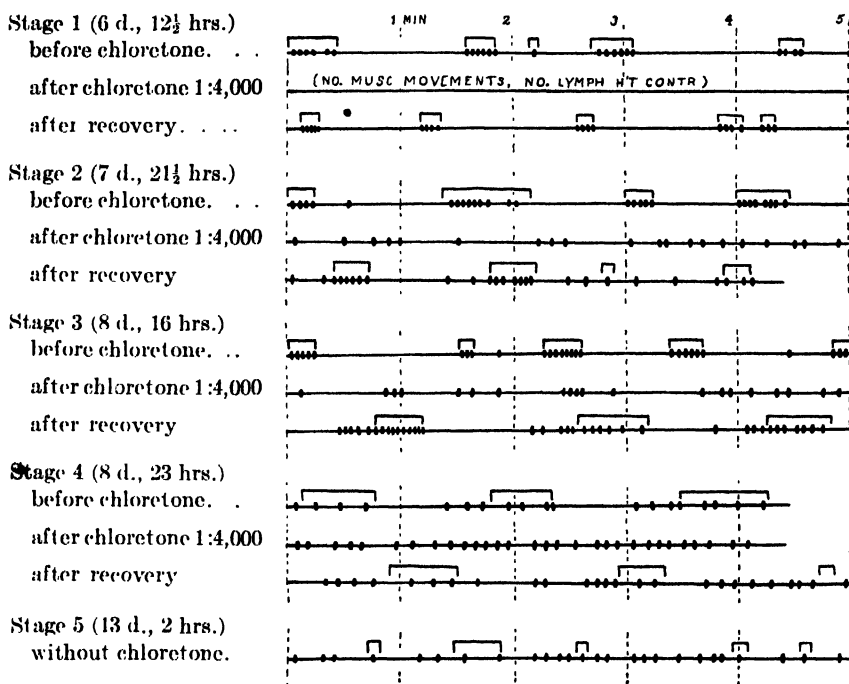


Chart 1 Shows graphically the relation between the lymph heart beats and body movements in different stages, before and after chlorethane and after recovery from chlorethane. The brackets — indicate the duration of the periods of body movement, the spaces between the brackets are the intervals of rest, and the dots are lymph heart pulsations. Selected portions of actual records are reproduced. Spaces between successive beats, length of periods of body movements and of intervals of rest represent actual proportional time intervals.

six days, in which the lymph heart pulsations, although distinct, are not yet very strong, the lymph heart contracted only once when stimulated directly by puncture with a glass needle. In the older embryos of Stage 1, puncture of the lymph heart caused it to beat six or eight times in succession and these beats were accompanied by tail and body movements. If the chick was anaesthetized at the time of puncture, each beat was accompanied by a slight mechanical pulling of the tail.

This early stage of the beating lymph heart, in which the pulsations occur only at the time of the periodic spasms of body

	OBSERVATION TIME	AVERAGE DURATION OF PERIODS OF BODY MOVEMENTS	AVERAGE TIME FROM BEGINNING OF ONE MOVING PERIOD TO BEGINNING OF NEXT	AVERAGE NUMBER OF Lymph HEART BEATS WITH EACH PERIOD OF MOVEMENTS	AVERAGE NUMBER OF Lymph HEART BEATS IN EACH INTERVAL OF REST	AVERAGE NUMBER OF Lymph HEART BEATS PER MINUTE
Stage 1 (6 d., 12½ hrs.) (GL. 20 mm.)						
before chloretone ..	45' 52"	13"	54"	3.3	0	3.8
after chloretone ..	15'	none			0	
after recovery ..	16'	11"	1' 06"	3.8	0	3.3
Stage 2 (7 d., 21½ hrs.) (GL. 24½ mm.)						
before chloretone ..	1°12' 50"	20"	1' 06"	5.3	1/9 ⁽¹⁾	5.1
after chloretone ..	7' 20"	none				4.7
after recovery ..	20' 10"	not rec.	1' 18"	5.6	1.8	5.1
Stage 3 (8 d., 16 hrs.) (GL. 27 mm.)						
before chloretone ..	37' 50"	not rec.	1' 13"	6.3	4/5 ⁽¹⁾	6.7
after chloretone ..	11' 22"	none				4.3
after recovery ..	17' 34"	not rec.	1' 57"	6.8	5.6	6.8
Stage 4 (8 d., 23 hrs.) (GL. 29 mm.)						
before chloretone ..	16' 28"	28.5"	1' 23"	3.4	2.6	4.5
after chloretone ..	18' 12"	none				8.0
after recovery ..	7' 8"	19"	1' 31"	not rec.	5.0	5.0
Stage 5 (13 d., 2 hrs.) no chloretone ..	18' 51"	not rec.	1' 08"	1.6	3.4	4.8

Chart 2 Shows the results of estimations as to average duration of periods of movement, etc., before, during and after the use of chloretone, at the various stages. The records used for this chart are the ones from which the selections in chart 1 were made: (1) (Stage 2) means that approximately one lymph heart pulsation occurred in each nine periods of rest; (1) (State 3) means, similarly that in each five periods of rest four single lymph heart pulsations occurred.

movements (unless the heart is actually punctured), was not observed by Budge, Sala, or Mierzejewski. These investigators all state that no contractions of the lymph heart occur before the ninth day of incubation. In all probability, this is due to the fact that the eggs, which they used, were opened in water and submitted at once to unfavorable conditions of temperature. In such a case the periodic movements of the embryo would cease

at once, and, with them, the pulsations of the lymph heart in chicks of this stage.

In chicks of seven to seven and one-half days (measuring 22 to 24 mm.) our records show practically the same connection between the beating of the lymph heart and the body movements. The lymph heart beat from three to nine times during each periodic spasm and never in the intervals of rest. But, at this stage, strong lymph heart contractions were accompanied at times by feeble tail movements and occasionally they were dissociated altogether from contractions of the tail.

Stage 2. Our observations of chicks of eight days showed that, on the average, 5.3 beats of the lymph heart accompanied each periodic spasm but that the majority of these beats were dis-coördinated from the tail movements. In addition, an occasional single pulsation of the lymph heart occurred in the period between spasms, with no accompanying body movement. Injections of the lymph heart at this and later stages, show it to possess the form of a definite oval sac.

One embryo of this stage (7 days, 21½ hours, measuring 24.5 mm.) was kept under continuous observation for four hours, and showed an interesting reaction to the treatment with chloretone. The chick was first observed for an hour and a half. We found that an average of 5.3 lymph heart beats accompanied each spasm and that, occasionally, an independent lymph heart contraction occurred in the period of rest. Chloretone (1:4000 in warm Ringer's solution) was then dropped on the embryo. The body movements and lymph heart beats both ceased for a few minutes. Then the lymph heart began to beat, an average of 4.7 times in every minute, with no accompanying body movements whatever. This beating of the lymph heart continued for forty minutes before the effect of the chloretone wore off. With the elimination of the body movements, the lymph heart pulsations occurred singly, instead of in groups, and they lost all semblance of periodicity.

Finally, after about fifty minutes, the body movements began to recur, gradually regaining their former periodicity. With the return of the body movements, the lymph heart regained its

former habit of beating approximately five times during each periodic spasm. However, it did not return completely to its previous mode of functioning for it still continued to contract from 2 to 4 times, independently, at irregular intervals during the period of rest. The observation was continued for an hour after the return of the body movements to their normal periodic rhythm and the lymph heart continued to contract in this same manner (charts 1 and 2: Stage 2).

Stage 3. Chicks of eight and one-half to nine days (27 to 29 mm.) resembled those of Stage 2, except for a slight increase in the independence of the beating lymph heart. An average of six pulsations took place periodically at the time of the body movements, but an isolated beat occurred in nearly every period of rest. Chloretone destroyed the periodicity of the lymph heart beats, which then occurred irregularly, about four or five to a minute. After the recovery from the anesthesia and the return of the body movements, the lymph heart again averaged six beats during each periodic spasm. In addition, it contracted about five times in every period of rest and these isolated beats, as a rule, preceded the period of body movements (charts 1 and 2: Stage 3).

When an embryo of Stage 3 or 4 was anesthetized and the lymph heart punctured by a fine needle, the lymph heart contracted six or eight times in succession, with no accompanying tail or body movements.

Stage 4. In chicks of nine to nine and one-half days (29 to 30 mm.) we found that the lymph heart still showed a tendency to beat periodically, but that its rhythm had now become somewhat discoordinated from that of the body movements. Although the periodic spasms in this stage were as long as or longer in their duration than those of younger embryos, the number of lymph heart beats occurring at this time was found to be less, an average of three beats instead of six. The number of isolated single beats occurring between spasms, however, was increased to three or four per interval. The isolated beats, as a rule, preceded the periods of body movements and, immediately after each spasm, there was a period of rest in which neither

body movements nor lymph heart beats were observed. When the body movements were paralyzed by chloretone, the groups of periodic lymph heart beats ceased and an increased number of single pulsations occurred, at irregular intervals. There were approximately eight beats per minute (charts 1 and 2: Stage 4).

Stage 5. Finally, in embryos of 10 to 13 days, the lymph heart contractions were found to be quite independent of the periodic body movements. At this stage, there was no semblance of periodicity in the pulsations of the lymph heart. Beats were seen to occur during the periodic spasms, but the intervals between such beats were not shorter than between those occurring in the period of rest. On the other hand, we observed several spasms during which no lymph heart beats whatever occurred (charts 1 and 2: Stage 5).

After the recovery from chloretone in Stages 2, 3 and 4, although the isolated number of heart beats was always greater than those occurring before the anesthesia, the total number of beats per minute remained practically the same. Also the number of beats per minute occurring during the period of anesthesia was not materially changed from the number originally present, in each of these stages (see chart 2). Although the chloretone dissociates the lymph heart pulsations from the body movements and greatly alters the rhythm in Stages 2, 3 and 4, it does not change, to any noticeable degree, the total number of lymph heart beats. Stage 1 is the only stage in which the function of the lymph heart is seriously interfered with, by the addition of chloretone, for in this stage only is it inseparably connected with the functioning of the voluntary muscular apparatus.

SUMMARY

We find, then, that pulsation of the lymph heart first appears in embryos of six to seven days (19 to 22 mm.) and that, at this earliest stage, the beating of the lymph heart is intimately connected with the periodic muscular movements of the embryo. Lymph heart contractions accompany the periodic spasms, never occur in the interim between body movements and each beat is invariably accompanied by a contraction of the tail. When

the body movements are paralyzed by chloretone, the lymph heart pulsations also cease.

In successive later stages, we find a gradual increase in the independence of the beating lymph heart. First, the pulsations become dissociated from the tail contraction, although still occurring only during the periods of body movements. Then we find a series of stages in which the lymph heart contracts more and more frequently during the period of rest, although still beating a number of times during each spasm. When chloretone is added, at these stages, a larger number of single pulsations occur independently, at irregular intervals. At these stages, the lymph heart is capable of entirely independent function, but is influenced, in its rhythm by the periodic spasms of body movements.

Finally, a stage is reached in which the lymph heart pulsations are uninfluenced, in any way, by the body movements. During all of these stages, mechanical stimuli, such as pressure over the surface with a fine needle, or direct puncture of the myotomes, failed to influence the body movements. Although the lymph heart does not respond to pressure over the surface, it always contracts when its wall is actually pierced. At the stage in which the lymph heart is not yet independent, such a puncture instigates tail contractions along with the lymph heart pulsations, while at later stages it stimulates the lymph heart alone.

DISCUSSION

In connection with our observations of the early pulsations of the lymph heart, we made serial sections of all the stages studied. In every case, the embryo sectioned was one which had been observed in the living and in which the movements and lymph heart pulsations had been recorded. From the study of these cross sections, however, we were unable to discover a definite anatomical relationship corresponding to the close functional association which exists, at first, between the lymph heart and the body musculature.

The following possibilities have suggested themselves in the course of our observations. It is possible:

1. That the lymph heart musculature is derived from the myotomes and that there is for a time a connection between the two.
2. That the muscle of the lymph heart is temporarily supplied by the same nerves which supply the myotomes.
3. That the lymph heart is merely stimulated mechanically by the contraction of the nearby muscles.
4. That, at first, the contractions of the lymph heart result from the same stimulus which brings about the periodic body movements, and that this stimulus is gradually subordinated to the stimulus caused by the increasing amount of lymph, brought to the lymph heart by the lymphatic vessels from the fast growing allantois and from the posterior half of the chick.

As regards the derivation of the musculature of the lymph heart from the myotomes, we have been unable to reach a definite conclusion from sections alone. In five-day chicks, the ventral extension of the myotome lies medial to the lymph heart plexus, separated by two or three layers of mesenchyme cells. During the succeeding day, this lateral portion of the myotome, which lies between the dorsal and ventral parts, becomes thinner, and in chicks of seven days and a few hours, only a few small bundles of somewhat isolated muscle cells remain. In sections of embryos of six and one-half days, with the oil immersion lens, we have seen muscle fibrillae in the wall of the lymph heart, which, at this stage, still retains the form of a plexus of lymphatic vessels. The muscle of the lymph heart, therefore, is present at an earlier stage than was supposed by Sala, who asserts that only mesenchyme is visible around the sac in embryos younger than eight and one-half days. In chicks of six and one-half to seven days, which is the stage at which the lymph heart beats always synchronously with the body movements, the wall of the lymph heart lies in close proximity to the thin lateral portion of the myotome already referred to. But, as regards the presence of a definite connection between the muscle fibrillae in the wall of the lymph heart and those composing this transitory portion of the myotome, we were unable to reach a positive conclusion.

In the matter of a common nerve supply for the myotomes and the lymph heart, our study of sections yielded only negative results, since we were unable to find any nerves in the wall of the lymph heart at any of the stages studied. However, further study with special nerve stains is necessary before this possibility can be excluded altogether.

We have already mentioned the fact that, during the earliest stage of its pulsation, the lymph heart lies in close proximity to the transitory lateral portion of the myotome. It is quite possible, therefore, that the contraction of the myotome, at this earliest stage, might stimulate the muscle of the lymph heart to contract, merely mechanically, without the presence of an actual connection between the two. In chicks of eight to nine days, with the disappearance of this part of the myotome, the lymph heart comes to occupy an isolated position in the mesenchyme. At this stage, however, the pulsation of the lymph heart is still influenced in its rhythm by the periodic contractions of the embryo. In an embryo of eleven days, the lymph heart has shifted its position and it now lies in close proximity to the dorsal muscle. But in this stage, in spite of its situation, the lymph heart is entirely independent in its beating, from the contraction of the rest of the musculature. Therefore, although the phenomena of the earliest stage of the lymph heart pulsation might be accounted for by the proximity of the myotome, which by its contraction stimulates the muscle of the lymph heart directly, the gradual manner in which the lymph heart pulsation becomes dissociated from the body contractions cannot be explained in this way. For, unlike the change in their physiological relationship, the change in the relative position of the myotome and the lymph heart muscle is sudden and not gradual.

In injecting the superficial lymphatics in living chicks, we have often observed that the lymph heart contracted as the fluid entered it. It is therefore capable of reaction to a stimulus from within as well as to the mechanical stimulation of its wall, and the gradually increasing independence of the lymph heart pulsations from the contractions of the body musculature may be

due to the growing intensity of the stimulus from within, occasioned by the constantly increasing flow of lymph. But further histological and experimental investigations are necessary for the complete explanation of the early pulsations of the lymph heart in chick embryos.

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STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN THE DOMESTIC FOWL. VIII. ON SOME PHYSIOLOGICAL EFFECTS OF LIGATION, SECTION, OR REMOVAL OF THE OVIDUCT¹

RAYMOND PEARL AND MAYNIE R. CURTIS

For several years there have been in progress at this Laboratory experiments dealing with various phases of the physiology of egg production. Among the reports of these investigations already published are several which deal with the physiology of the oviduct. A few of the more important of the results already obtained may be reviewed briefly here, in order to show more clearly the relation to them of the present findings.

1. Albumen secretion is not confined, as was formerly supposed, to the albumen secreting region of the oviduct, but also takes place in the isthmus and uterus. It is in fact probably a general function of the entire oviduct (Pearl and Curtis '12, and Surface '12).

2. There is some good evidence that for any part of the oviduct the effective stimulus to secretion is mechanical. Tarchanoff ('84) and Weidenfeld ('05) showed that a normal set of egg envelopes including chalazae, albumen, egg membrane and shell may be formed around an artificial yolk (wood, rubber, or amber bead). This result has been confirmed at this Laboratory with artificial yolks of agar-agar. Our experiments on this point have never been described in detail. The results were briefly stated in a previous paper (Curtis '14b). These experiments also agree with those of Tarchanoff that this result does not always follow the introduction of an artificial yolk into an active oviduct. The other necessary conditions

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¹ Papers from the Biological Laboratory of the Maine Agricultural Experiment Station, No. 68.

are not yet fully understood. Pearl and Surface ('09 a) published a short report of a preliminary investigation of the stimulus which sets up the shell secreting activity. They showed that when the intestine was anastomosed to the oviduct above the uterus the faeces extruded through the vagina or retained in the uterus were covered with a deposit of shell material. They concluded that the immediate effective stimulus to shell secretion is mechanical and that shell formation is a local reflex not dependent upon a specific activity of other parts of the reproductive apparatus.

3. The amount of secretion by the duct depends in part upon the intensity of stimulation. Within the eggs of the same individual the weight of albumen is highly correlated with the weight of the yolk and the weight of shell is significantly positively correlated with the weight of both albumen and shell (Curtis '14 a). In yolkless, single, double and triple-yolked eggs the weight of albumen and shell are directly related to the number of yolks contained (Pearl '10, and Curtis '14 b).

4. The muscular activity of the walls of the oviduct are undoubtedly responsible for the shape of the egg (Pearl '09, and Curtis '14 b). Further this muscular activity is capable of regulatory changes. Pearl ('09) described the case of a pullet whose first eggs were much longer in proportion to their breadth than any other eggs produced at the Maine Station plant. There was a progressive regulatory change in the eggs of this bird until they finally became normal in shape.

5. The resection of one-fourth of the albumen secreting region and an end-to-end anastomosis of the remaining parts does not cause a permanent loss of function of the oviduct. Pearl and Surface ('08) described the results of such an operation. The bird began to lay four months after the operation. The eggs were only a little below the average size for the breed and appeared to have a normal proportion of albumen. There seems here also to be a compensatory action of the duct so that the amount of secretion is nearly normal. Whether there is an increase in the number of albumen glands or in the functional activity of those left is not known.

The experiments already described were confined to a study of the physiological processes of the oviduct itself. In all cases the functional integrity of the duct was preserved. During the course of the investigations into the physiology of reproduction, however, several operations have been performed where egg production has been prevented by the removal of a part or all of the oviduct, or by closing it at various levels. The purpose of the present paper is to show the results of such interferences upon, first, the growth of the ovary, second, the secondary sexual characters, third, the body metabolism, and fourth, the growth and ability to function of any remaining portion of the oviduct itself.

TECHNIQUE

The technique used in these operations was a simplified form of the technique of aseptic human surgery. A bird was given a subcutaneous injection of 1/200 grain atropine sulphate in 1 cc. of normal salt solution. Just sufficient ether was given to maintain complete insensibility (Pearl and Surface '09 b). On the bird was placed a clean muslin jacket which held the wings close to the body and covered most of the feathers. The feathers were picked off the field of operation and the skin painted with iodine.

The top of the operating table used is an especially constructed adjustable slat trough. This trough is adjusted so that when the back of the bird is at the bottom of the trough the ventral surface is even with the edges. The bird is secured to this table by means of strings attached to the legs and wings.

A sterile muslin screen attached to an iron hoop at one end of the table is stretched back over the bird. A hole is made in this screen in the proper place to expose the field of operation. This screen makes the bird's head accessible to the person giving the ether and yet shuts off this source of contamination from the field of operation. Sterile towels are placed above the screen so as to cover deeply the whole bird except the iodine

painted field of operation. Care is taken to have all instruments, sponges, and ligatures thoroughly sterilized.

The abdominal incision for operations on the oviduct is to the left of the middle line. The intestines are crowded to the right and covered with a piece of sterile vaselined silk and held with a retractor. The oviduct is thus exposed. After the operation the muscle incision is closed by several interrupted sutures with fine silk and the skin incision by continuous sutures with catgut. The closed incision is finally painted with collodion. In almost all the cases described the wound healed quickly by first intention and the stitches in the skin were removed about a week after the operation. In a few cases there was a slight granulation of the skin wound.

THE EFFECT OF INTERFERENCE WITH THE OVIDUCT UPON THE GROWTH OF THE OVARY, AND ITS FUNCTIONAL INTEGRITY

For many years it was an apparently generally accepted notion that the section or removal of a bird's oviduct caused degeneration of the ovary. Yarrell ('27) says that if a small portion of the oviduct is removed the ova do not afterward enlarge. According to Tegetmeier ('76) 'poulardes'² are fattened females rendered sterile when young pullets by cutting the oviduct, or by removing a portion of it. In regard to the effect of this operation he makes the following statement: "The development of the ovary or egg-producing organs is entirely prevented and the birds fatten rapidly attaining also a large size." Bland Sutton ('85) and Brandt ('89) also state on Yarrell's authority that removal of the oviduct causes the shrinking of the ovary.

It is well known from gynecological practice that in man the section or removal of portions of oviduct does not cause atrophy of the ovary. McIlroy ('12) showed that in rabbits, rats and guinea pigs removal of the ovary caused atrophy of the uterus, mammae, and external genitals, but removal of the uterus had no effect on the ovary. Sellheim ('07) has shown

² The term is still used in France, but is now in reality applied to any very fat female especially to a very fat pullet. They are often said to be sterilized females.

that in the domestic fowl also removal of the oviduct does not cause a permanent degeneration of the ovary. He states that the ovary at first shrinks but later is in the same condition as in unoperated birds. The yolks are discharged into the body cavity.

The results of twenty-nine operations on the oviduct are given in table 1. In twenty-four of these cases (all except 21 to 24 and 27) egg-laying was prevented. In fifteen (1 to 9 and 15 to 20) yolks could not enter the duct. In cases 15 to 20 practically the entire duct was removed when either in infantile or non-laying condition. The birds were autopsied at periods varying from ten days to nearly five years after the operation. They were in varying conditions of health and disease. Their ovaries presented the same range of variation shown in our large series of autopsy records on unoperated birds. Those of the normal healthy birds showed all stages from strictly non-laying to fully functional condition, while several of the birds with serious visceral lesions showed various stages of absorption of ovarian yolks. In no case is there the slightest evidence of atrophy of the ovary.

Several of the operations especially on the removal of the oviduct (cases 15 to 20) were performed before the sex organs had begun the rapid growth which precedes the first period of production in the pullet. In all of these cases the ovary continued to grow.

These results show that neither the ligation, section, nor entire removal of the oviduct causes degeneration or prevents the further growth of the ovary.

Sellheim ('07) states that after the removal of the oviduct the ovary at first shrinks. He believes this to be a result of the severe operation. Our experience shows that a bird is not in laying condition for some time after any serious abdominal operation involving prolonged anesthesia and considerable surgical shock. It seems quite possible that the 'shrinking' of the ovary noted is the return to a strictly non-laying condition due to a general physiological disturbance rather than the specific effect of the removal of the oviduct. This post-operative

TABLE 1

Showing the results of the ligature, section or removal of the oviduct

CASE NO.	OPERATION NO.	DATE OF OPERATION	AGE AT OPERATION	CONDITION OF OVIDUCT AT OPERATION	NATURE OF OPERATION	CAUSE OF DEATH	DATE OF AUTOPSY	BODY WEIGHT AT OPERATION: GRAMS	BODY WEIGHT AT AUTOPSY: GRAMS	GAIN IN WEIGHT: GRAMS	LENGTH OF TIME AFTER OPERATION	CONDITION OF OVARY AT AUTOPSY	CONDITION OF OVIDUCT AT AUTOPSY	REMARKS
1	84	Mar. 5 1909	9 mos.	laying	Funnel mouth closed with several interrupted sutures after insertion of artificial yolk (cork)	Internal bleeding from enlarged and ruptured spleen; some peritonitis	June 4 1909	2225	2872	447	3 mos.	Seven absorbing yolks all of which had apparently been large; 4 normal ones above 1 cm.; wt. of largest 10.5	Oviduct not very large but healthy; funnel still closed by sutures	Body fat; several small masses of hard yolk in body cavity
2	85	Mar. 5 1909	9 mos.	laying	Funnel ligated after the insertion of an artificial yolk (cork)	Killed for data	Mar. 18 1909	1970	2050	80	13 days	Several large normal yolks on ovary	Oviduct in laying condition	All abdominal conditions normal
3	87	Apr. 14 1909	10 mos.	laying	Funnel ligated after the insertion of an artificial yolk (agar)	Internal bleeding from abdominal fat	Apr. 24 1909	1840	1455	-385	10 days	Ovary in non-laying condition; no yolks above 3 mm.	Oviduct small; weight 9.8 grams	Utrates deposited on peritoneum covering viscera
4	95	Apr. 16 1909	10 mos.	laying	Funnel ligated after the insertion of an artificial yolk (one BB shot)	Killed for data	Apr. 29 1909	2070	1832	-138	13 days	Ovary normal and healthy with 6 large normal yolks	Oviduct in laying condition	A yolk walled off with peritoneum attached to funnel above ligature
5	97	Apr. 16 1909	9 mos.	laying	Funnel ligated after the insertion of an artificial yolk (clay marble)	Killed for data	Apr. 29 1909	1070	795	-275	13 days	Ovary in non-laying condition; a few small absorbing yolks were present	Oviduct resembled that of a bird which had not laid for 10 days to a month and without large yolks in ovary; glandular ridges deep	Bird in excellent condition; no infection; no adhesions

6	98	Apr. 16 12 1/2 mos. 1909	laying	<i>Funnel ligated</i> after the insertion of an artificial yolk (marble)	Killed for data Apr. 29 1909	840	770	-70 13 days	Ovary in non- laying condition; a few small ab- sorbing and a few small normal yolks	Oviduct in same condition as in case 5	Slight surface in- fection of wound; no internal infec- tion; no adhesions
7	7	Jan. 16 8 mos. 1908	laying	Removal of part of albumen por- tion of the oviduct and end-to-end anastomosis; later <i>funnel adhered to</i> <i>intestine some dis-</i> <i>tance behind ovary;</i> opening small and filled with a plug of hard yolk	Peritonitis Mar. 18 1910	2030	2030	2 yrs. 2 mos.	Ovary with three follicles; no egg with yolk	Oviduct in lay- ing condition and some granular se- cretion was con- tained in the duct	Body cavity con- tained yolk masses and some semi- fluid yolk; bird had laid from March 17, 1908, to Oct. 6, 1909
8	96	Apr. 16 10 mos. 1909	laying	The ligature which was placed around the funnel after the insertion of an artificial yolk was found at autopsy untied but a large hyaline tumor was at- tached to one lip of the funnel mouth and the other lip was closely pressed against the tumor. <i>Oviduct was there-</i> <i>fore closed at the</i> <i>funnel mouth</i>	Killed for data May 10 1909	2180	2260	80 24 days	Four large yolks on ovary; wt. of largest 14.0	Oviduct in lay- ing condition; wt. 54 grams	Wound healed. No infection and no adhesions; fresh- ly broken yolk in body cavity

TABLE 1—Continued

CASE NO.	OPERATION NO.	DATE OF OPERATION	AGE AT OPERATION	CONDITION OF OVIDUCT AT OPERATION	NATURE OF OPERATION	CAUSE OF DEATH	DATE OF AUTOPSY	BODY WEIGHT AT OPERATION: GRAMS	BODY WEIGHT AT AUTOPSY: GRAMS	GAIN IN WEIGHT: GRAMS	LENGTH OF TIME AFTER OPERATION	CONDITION OF OVARY AT AUTOPSY	CONDITION OF OVIDUCT AT AUTOPSY	REMARKS
9	16	Oct. 28, 1908	6 mos.	infantile	Oviduct ligated at level of the shell gland	Killed by other birds picking open body cavity at anus	Dec. 29, 1908	1560	2219	659	2 mos.	Three large yolks on ovary	In functional condition with two normal eggs in the duct just above the ligature; both these eggs had some shell	Body fat; wound completely healed; 5 normal and 2 yolkeless eggs in body cavity; 3 of the normal eggs were surrounded with peritoneum and were in varying stages of absorption; these eggs had considerable shell; another half hard shelled egg was only partly surrounded by peritoneum and was not perceptibly absorbed; the other normal and the two yolkeless eggs were without shell, were not surrounded by peritoneum and were not apparently absorbing

10	36	Nov. 1264 mos. 1908	infantile	Oviduct ligated at level of shell gland	Killed by other birds picking open body cavity	Mar. 7 1910	1326 16 mos. After (See note in viscera pre- ced- been ing eaten col- out by umn) other birds.	Seven large normal yolks on ovary.	Body fat; viscera eaten out by other birds except the ovary and a part of a yolk mass enclosed in peritoneum	
11	64	Feb. 16 9 mos. 1909	laying	Resection of isthmus and end-to-end anastomosis; at autopsy it appeared that nothing had passed the anastomosis which was therefore virtually a ligature	Internal bleed- ing from ruptured spleen	Apr. 20 1909	2110 grams	2 1/2 mos. Large yolks on ovary but they were all hard	Abdominal viscera a mass of adhesions	
12	68	Feb. 18 9 mos. 1909	laying	Reversal of polarity of a portion of isthmus and albumen portion; the piece was resected, turned end-for-end and anastomosed back into the duct; at autopsy duct was found shriveled and passage closed just below first anastomosis	Peritonitis	Mar. 11 1913	4 yrs., 1 mo.	Not noted	Very large and filled with hardened egg masses; some of these were in the reversed portion but apparently none had passed the second ligature; shell gland and vagina were empty	Many yolk masses in body cavity surrounded by peritoneum and attached to varying peritoneal surfaces

TABLE 1—Continued

CASE NO.	OPERATION NO.	DATE OF OPERATION	AGE AT OPERATION	CONDITION OF OVIDUCT AT OPERATION	NATURE OF OPERATION	CAUSE OF DEATH	DATE OF AUTOPSY	BODY WEIGHT AT OPERATION: GRAMS	BODY WEIGHT AT AUTOPSY: GRAMS	GAIN IN WEIGHT: GRAMS	LENGTH OF TIME AFTER OPERATION	CONDITION OF OVARY AT AUTOPSY	CONDITION OF OVIDUCT AT AUTOPSY	REMARKS
13	17	Oct. 29, 1908	5 mos.	infantile	Section of the duct. (tube cut across between two ligatures)	Abdominal hernia and subsequent infection and peritonitis	Mar. 13, 1909	1390	2640	1150	5 mos.	Ten yolks on ovary; some hard; all abnormal	Both parts of the oviduct had enlarged to laying condition; no egg masses in duct but there was a lump of hard yolk just above the ligature, i.e., at end of first piece of the duct	Body fat; 5 yolks in body cavity enlarged off by peritonsium; peritonitis appeared due to infection from hernia
14	37	Nov. 13 ⁷⁴ , 1908	13 ⁷⁴ mos.	infantile	Oviduct was cut across through the shell gland	Killed for data	June 21, 1909	1400	2294	894 ⁷⁴	mos.	Ovary in laying condition; 10 follicles varying in size from large to just viable ones; 3 large normal yolks; wt. largest 13.4 grams	Oviduct in laying condition; punctured 13 cm. above upper ligature; duct filled with egg masses made up of non-absorbed and 1 small membrane covered absorbing eggs; calcareous mass size of a common red cherry in shell gland; part of shell gland and vagina behind the cut were the size of these parts in a laying bird; vagina open into cloaca	Body fat; in body cavity were 9 empty egg membranes, 1 with a small amount of egg substance still perfectly fresh non-absorbed and 1 small egg with germ disk visible through membrane. Metabolism of bird seemed O.K.; no sign of peritonitis or putrefaction of egg masses

15	18	Oct. 30 5 mos. 1908	infantile	Oviduct removed except the part of the vagina applied to the colon and the portion of the fused lips dorsal to the abdominal air sac	Killed for data	Aug. 9 1909	2206	1206	9 mos	Ovary in normal condition; 6 yolks above 1 cm.; left at the operation quite large ruptured follicle	The small portions of the duct left at the operation had enlarged to the normal size of these parts in a laying hen; oviduct open into cloaca	Body very fat; no unabsorbed yolk in body cavity
16	34	Nov. 13 5 mos. 1908	infantile	Oviduct removed with the exception of the part of the vagina closely applied to cloaca	Unknown	June 14 1909	1480	1995	515 7 mos.	Ovary with 5 small absorbing yolks and some small normal ones; wt. with yolks 4.5 grams	Only the part of vagina attached to the colon present	Hard masses evidently yolk free in body cavity
17	35	Nov. 13 5 mos. 1908	infantile	Oviduct removed with the exception of the part of the vagina closely applied to the cloaca	Killed for data	Aug. 24 1911	1000	3769	2 yrs., 9 mos.	Ovary with several large yolks; also several absorbing	Oviduct all gone except vagina which was the size of the vagina in a laying hen	Body very fat; no yolks in body cavity
18	42	Nov. 20 1 1/2 yrs. 1908	non-laying but adult	Oviduct removed except funnel and vagina	Stoppage of intestine due to adhesions	June 23 1909	2150	1939	7 mos.	Yolks on ovary hard	All gone except funnel portion; presence or size of vagina not recorded	Body cavity filled with yolk masses covered with peritoneum
19	45	Nov. 24 6 mos. 1908	Beginning to enlarge for first laying period; about 15 cm. long	Oviduct removed except funnel, part of shell gland and vagina	Tuberculosis	July 14 1913	1800		4 yrs., 8 mos.	Many had partly absorbed yolks on ovary	Part of shell gland and vagina in condition of a bird which had lately been laying; size of funnel not recorded	Body cavity contained a large number of absorbed yolks enclosed in peritoneum
20	51	Dec. 3 7 mos. 1908	infantile	Oviduct removed entire	Killed because she was sick	Feb. 23 1909	1070	1665	595 1 yr., 2 mos.	In healthy non-laying condition	Oviduct found	Condition of other organs not recorded

TABLE 1—Continued

CASE NO.	OPERATION NO.	DATE OF OPERATION	AGE AT OPERATION	CONDITION OF OVIDUCT AT OPERATION	NATURE OF OPERATION	CAUSE OF DEATH	DATE OF AUTOPSY	WEIGHT AT OPERATION: GRAMS	WEIGHT AT AUTOPSY: GRAMS	GAIN IN WEIGHT: GRAMS	LENGTH OF TUBE AFTER OPERATION	CONDITION OF OVARY AT AUTOPSY	CONDITION OF OVIDUCT AT AUTOPSY	REMARKS
21	12	Oct. 22 1908	8 mos.	laying	Lumen of the abdominal region of duct diminished by taking a tuck in the muscular coat 14 cms. long	Killed for data	May 13 1909	1280	2125	845 g.	8 mos.	In laying condition; 6 yolks on ovary; wt. of largest 15.5	In laying condition; no sign of the tuck; soft shelled egg in shell gland	Bird had been laying since Jan. 17, 1909
22	72	Feb. 16 1909	11 mos.	laying	Lumen of abdominal region of duct constricted by a tuck 44 cms. long	Killed for data	May 14 1909	1530	1177	-353	3 mos.	In laying condition; 4 normal yolks above 1 cm.; wt. of largest 15.0 grams	Tuck was preserved in outer layer but it made the lumen of the duct; egg at entrance of isthmus	Bird laid a normal egg Mar. 13, 1909
23	62	Feb. 16 1909	8 mos.	laying	Diminishing size of shell gland by removal of piece of the wall	Killed for data	May 14 1909		2140		3 mos.	In laying condition; 4 large yolks on ovary; wt. of largest 14.5 grams	Oviduct in laying condition; shell gland of normal shape although possibly a little small; silk still in tissue where cut had been sewed	Bird has laid since Mar. 16, 1909; eggs about average size for breed
24	65	Feb. 17 1909	8 mos.	laying	Longitudinal incision of shell gland by interrupted sutures	Killed for data	May 14 1909	1600	1505	-95	3 mos.	In laying condition; 4 large normal yolks on ovary; wt. of largest 10.5	In normal laying condition; pieces of silk found in wall of shell gland; these did not appear to be tied; no appearance of tuck internally or externally	Bird had been laying since Mar. 13, 1909; eggs below average size for the breed but not below range of breed variation

25	75	Mar. 4 9 mos. 1909	laying; egg left in shell gland	Ventralligament cut where it is a round mass of muscle on ventral side of shell gland; funnel also ligated	Bird was acci- dentally sold in summer 1910					Laid the egg which was in snell gland at the time of operation on the day following; did not lay again		
26	109	Oct. 24 3 mos. 1909	Infan- tile	Removal of en- tire ventral liga- ment	Killed because of peritonitis	June 4 1910	1490	1376	886 $\frac{3}{4}$ mos.	Several large ab- sorbing yolks on ovary	Ventral ligament lacking; duct size laid but had ova- ry of a duct of a bird which had laid 10 days previously; vagina open to cloaca	Bird had never laid but had ova- ry of a duct of a bird which had laid 10 days previously; vagina open to cloaca
27	112	Nov. 4 $\frac{1}{2}$ 1909	infan- tile	Piece of the ven- tral ligament $\frac{1}{2}$ cm. long removed	Killed because of peritonitis	Feb. 24 1910	1620	2093	473 $\frac{1}{4}$ mos		Ventral ligament lacking through- out the length of the isthmus but normal at upper end and there was a hard mass of muscle along the ventral side of shell gland	Had laid several normal eggs
28	113	Nov. 4 7 mos. 1909	infan- tile	Piece of the ven- tral ligament 1 cm. long removed	Killed	Dec. 7 1910	1900	1039	339 13 mos	In non-laying condition	Not in laying condition; ventral ligament at head of shell gland in laying condition; viscera nor- mal	Had not laid; no evidence that the bird had ever been in laying condi- tion; viscera nor- mal
29	119	Nov. 18 4 mos. 1909	infan- tile	Piece of the ven- tral ligament 0.7 cm. long removed	Digestive dis- orders	Feb. 11 1910	435	429	-6 4 mos.	In non-laying condition	Not in laying condition; a part of the ventral liga- ment along the shell gland want- ing	Had not laid and there is no evidence of the bird had ment along the shell gland want- ing

'shrinking' no doubt accounts for the belief of the early workers that extirpation of the oviduct caused degeneration of the ovary. Our experiments on the removal of the oviduct do not bear upon this point since at the time the ducts were removed (cases 15 to 20) the sex organs were in infantile or non-laying condition and they were not examined for at least seven months after the operation. Cases 2 and 5, however, show that the ovary may be in full functional condition 13 days after the funnel is ligated.

THE EFFECT OF INTERFERENCE WITH THE OVIDUCT UPON THE SECONDARY SEXUAL CHARACTERS

The early workers Yarrell ('27), Bland Sutton ('85), and Brandt ('89) state that the removal of the oviducts is followed by the assumption of male secondary sexual characters by the female. The belief of the early workers that removal of the oviduct caused the degeneration of the ovary no doubt led them to misinterpret the normal growth of the comb, wattles, and spurs, as the assumption of male characters.

None of the birds from which we removed or otherwise interfered with the oviduct showed any tendency to assume male characters. At the time of autopsy they were as female in all secondary sexual characters as normal birds. This result also agrees with those of Sellheim ('07). He summarized the results of his experiments as follows:

Damit ist für die Henne der Beweis geliefert, dass die Entfernung des Legdarmes keine schädliche Wirkung auf die Funktion des Eierstockes ausübt. Dass die Legdarmresektion in ihrer Wirkung einer Kastration gleich setzen sei und dass die sekundären Geschlechtscharaktere, Bartläppchen, Kämme, Sporen, Gefieder, Beckenform, Stimme, benehmen gegen das andere Geschlecht nach der Exstirpation des Legdarmes sich ändern, ist reines Phantasiestück.

THE EFFECT OF THE LIGATION OR REMOVAL OF THE FUNNEL UPON OVULATION

Coste ('74) describes the infundibulum embracing the ovarian yolk in its follicle directly before the time for ovulation. The same phenomenon has since been observed many times by other workers, including the authors. So far as is known the in-

fundibulum has never been observed³ in the act of swallowing a free yolk. Further, the work of Patterson ('10) and Bartelmez ('12) shows that normally the follicular orientation is preserved in the oviduct. These considerations make it certain that normally the yolk is ovulated into the enclosing funnel.

Coste believed that the pressure of the funnel upon the follicle was the probable cause of ovulation and Patterson ('10) apparently accepts this view. The walls of the funnel are muscular and at the time it embraces the follicle it is in active peristalsis. That the pressure is sufficient to cause or at least materially to aid ovulation is a natural inference.

³ That yolks set free in the body cavity may subsequently enter an oviduct seemed the most reasonable explanation for the conditions observed in two birds (no. 17K and 397K) autopsied at this Laboratory. Both these birds were pure bred Barred Plymouth Rock hens a little over one year old and each had laid a number of eggs. Bird no. 17K had not laid for four months and bird 397K had not laid for one month. At autopsy the sex organs on the left side of the body of each bird were in practically non-laying condition. In neither bird was there any right ovary. Each bird had what appeared to be a right oviduct filled with large egg concretions. These concretions were exactly similar to such masses often found in abnormal conditions of the left oviduct. They appeared to consist of concentric layers of hardened albumen surrounding hardened yolks. In both birds the upper end of the tube was greatly distended. Its walls were stretched thin as is a left oviduct containing large egg masses. Smooth muscle fibers were visible in the walls. Each bird showed considerable peritonitis and there were adhesions between the walls of the tube (oviduct) and intestine. The funnel mouth could not be distinguished. The lips had apparently adhered together; (this is often true in the case of egg concretions in the left oviduct). The tube continued for several centimeters behind the caudal end of the masses. It ended blindly near the cloaca into which it did not open. There was no differentiated shell gland. In Bird no. 397K the tube had ligaments very much like normal oviduct ligaments. In Bird no. 17K the ligaments were not exactly like normal oviduct ligaments but the tube was held in a fold of peritoneum. There seemed no doubt that these egg masses were egg concretions formed in the rudimentary right oviducts and little doubt that the centre of these masses were yolks. If this was true the yolks must have been ovulated from the left ovary and have passed across the body cavity (behind the gizzard in Bird 17K and by this path or through a hole which was found in the mesentery in Bird 397K) and there have been picked up by the right oviduct. It should possibly be added that other large right oviducts have been observed in the routine autopsy work. One case (Bird no. 276) had both right and left oviducts in nearly functional condition. The typical parts were differentiated in the right duct and it was open into the cloaca. In this case also there was no right ovary.

Bartelmez ('12) has shown that in the pigeon continued yolk formation increases the pressure within the follicle so that "the egg bulges out when the rupture begins" and "the egg is over a millimeter in diameter greater after ovulation than the whole follicle was before." He and other workers recognize in this increase in internal pressure an important factor in the rupture of the follicle.

That the pressure of the infundibulum is not necessary for the rupture of the follicle is proven by the experiments of Sellheim ('07), which show that after the oviduct is removed ovulation takes place into the body cavity. This result is confirmed by the present investigation which shows that ovulation into the body cavity occurs when the funnel mouth is closed by sewing or ligating the ostium (cases 1, 4, 7 and 8); or by removal of the duct (cases 15, 16, 18 and 19). In cases 4, 8, 15 and 17 the birds were killed during a normal period of egg production and (with the exception of case 17) showed evidence of recent ovulation into the body cavity. In these birds none of the yolks remaining in the ovary were apparently larger than yolks found in the ovaries of normal laying birds of the same breed. Quantitative data are not as yet available on this point, but the observations indicate that absence of pressure exerted by the funnel does not perceptibly delay ovulation. This suggests not only that internal pressure is a sufficient cause for rupture of the follicle, but also that it may be the most important factor in causing such rupture in the case of normal ovulation into the oviduct.

THE EFFECT OF PREVENTION OF EGG-LAYING ON BODY METABOLISM

It has already been shown that if the oviduct is removed or if the mouth of the duct is permanently closed the ovary passes through normal periods of egg production and the yolks are ovulated into the body cavity. Cases 9, 10, 12, 13 and 14 show that when it is possible for a yolk to enter but not to leave the duct, the duct may be filled with egg masses and ovu-

lation then takes place into the body cavity or if the duct be stopped at the level of the shell gland normal eggs may be formed and passed back up the duct into the body cavity. The fate of these yolks and eggs and their effect on body metabolism is of some interest.

At the height of a period of egg production a bird is consuming a large amount of food materials which she is elaborating into eggs. In the cases cited she is discharging these products into her own body. Is she able to resorb these eggs and if so by what process is it accomplished? And are the resorbed eggs utilized in the body metabolism?

There are in all fourteen cases where the bird at autopsy showed that she had been either ovulating (cases 1, 4, 7, 8, 10, 12, 13, 15, 16, 18, 19 and 26) or backing fully formed eggs (cases 9 and 14) into the body cavity. Four of these fourteen (cases 1, 7, 12 and 26) died from peritonitis which may have been caused by inability to absorb the yolks in the body cavity. The other ten birds, or 71 per cent, were able to resorb the yolks or eggs. Moreover, in none of the four cases of peritonitis were we certain that it was due to the presence of the yolks although this seemed the most probable cause.

In addition to the fourteen cases cited in the preceding paragraph there are in the archives of the Laboratory autopsy records of other birds which at the time of death were ovulating into the body cavity and resorbing the yolks. Two methods of resorption are observed. First, absorption directly through the general peritoneal surface, and second, walling off of the yolks or eggs by peritoneum and subsequent absorption. In cases 1, 7, 8, 14, 15⁴ and 16 absorption was by the first method. None of these cases present such clear evidence of rapid absorption of several yolks as some cases met with in the routine autopsy work. Descriptions of two such cases follow.

Bird no. 1406 laid but seven eggs during her life. The last of these was laid August 22 of her pullet year. She was kept

⁴ In this case the yolk had been entirely absorbed but the follicle on the ovary was large and it therefore seemed impossible that sufficient time had elapsed for absorption by the second method.

until February 6 of her second year when she was killed for data. She was twenty-two months old and was a large and very fat bird. In the uterus was a small membrane-shelled egg weighing only 23 grams. The yolk of this egg weighed only 4.87 grams. The oviduct was normal except that the opening of the funnel seemed small. In the ovary were four yolks above one centimeter and six between a centimeter and a millimeter in diameter. The ovarian yolks were a normal series of maturing yolks, the largest of which was apparently too large to enter the oviduct. The ovary also contained four large follicles. In the body cavity was a creamy oily fluid. This was apparently yolk mixed with serum. There was no appearance of peritonitis; in fact the bird was apparently in perfect health.

Bird no. 1375 laid reasonably well during her first two laying years producing 141 eggs the first year and 117 the second but after the second adult molt she laid but one egg (February 14, 1914). On March 7, 1914, she was killed for data. She was thirty-five months old and was very fat. On opening the sheet of fat enclosing the viscera they were seen to be covered with thick fresh egg yolk. The peritoneum was perfectly normal. The oviduct was large and normal in appearance. The finger could easily be inserted into the funnel mouth which was apparently large enough to admit a normal mature yolk. The duct was open throughout. The glandular ridges of the albumen secreting and isthmus regions were expanded and whitish, as if full of secretion. There was a little albumen between the ridges in the albumen secreting portion. The funnel hung rather loosely from its ligamentary attachments. When the body cavity was opened the *ostium* was situated at some distance behind the largest yolk. The intestines were still capable of normal peristalsis and slight peristaltic movements could be induced in the oviduct. Quantitative data are lacking but the response of the duct seemed less vigorous than the normal response of the oviducts of laying birds when stimulated immediately after death. In the ovary was a large empty follicle apparently just discharged and five others ranging gradually in size from this to one about 1 mm. in diameter. There were also

very many which were just distinguishable. The ovary also contained a normal series of maturing yolks. Four of these were above 1 cm. in diameter. The largest was apparently mature.

These two birds then had normal ovaries and at the time of death were maturing and ovulating yolks at a rate comparable to that shown by birds at the height of a normal period of egg production. They also possessed oviducts which were apparently able to secrete the normal enclosing envelopes. The fact that the yolks did not enter the duct and become the yolks of normal eggs was probably due, in the first case, to the fact that the funnel mouth was too small to admit a full sized yolk, and in the second to a lowered state of tonus in the muscles of the oviduct and oviduct ligaments. They represent a type of non-production previously discussed by one of the authors (Pearl '12) where sterility is due to 'somatic' (physiological) rather than 'gametic' causes. From the point of view of the present investigation they show that a bird may ovulate into the body cavity and resorb the yolks from the general peritoneal surface at an astonishingly rapid rate without causing any apparent disturbance of normal metabolism.

In the second type of absorption in the body cavity the yolks or eggs are enclosed in separate sheets of peritoneum by which they are attached to the adjacent peritoneal surface. This type of absorption has been several times observed in routine autopsy work as well as in cases 4, 10, 12, 13, 18 and 19. Case 9 shows all the steps in the process.

This bird's oviduct had been ligated at the level of the caudal end of the shell gland before it has enlarged for its first period of egg production. The sex organs developed normally. The duct received the yolks and formed normal eggs which were discharged back through the funnel into the body cavity. At the time of autopsy the body cavity contained five normal and two yolkless eggs, and there were also two normal soft shelled eggs in the oviduct just above the ligature. The condition and position of the eggs in the body cavity were as follows:

- (1) A collapsed shell, slightly calcareous, containing a small

amount of mixed albumen and yolk was surrounded by peritoneum by which it was firmly attached to the abdominal fat and the right side of the colon at the posterior end of the body cavity. (2) A partly collapsed soft shelled egg, surrounded by peritoneum which attached it to the peritoneum covering the left kidney, lay between the left kidney and the oviduct just behind the ovary. (3) A soft shelled egg, surrounded by a very thin sheet of peritoneum, was attached to the right side of the intestinal mesentery just opposite the ovary. (4) A normal hard shelled egg occupied the posterior end of the body cavity to the left of the intestine. A part of the shell was covered with peritoneum. (5) A soft shelled egg not surrounded by peritoneum lay just behind 3, into the side of which it was indented. (6) Two yolkless membrane shelled eggs were free in the body cavity among the convolutions of the oviduct.

This bird was clearly discharging eggs into the body cavity at a rapid rate. These eggs were evidently moved about in the body cavity by the movements of the intestine and oviduct until they were pushed into some portion of the cavity where they remained quiet long enough for the local peritoneal surface to throw out a surrounding envelope of peritoneum. The four eggs attached or in the process of being attached were at the four corners of the abdominal cavity and were therefore in positions least likely to be affected by the movements of digestion and egg formation.

It sometimes happens that yolks become attached to the peritoneum forming the intestinal mesentery or the oviduct ligaments. Apparently if an egg or a yolk free in the body cavity is allowed to remain quietly in one position the adjacent peritoneum throws an enclosing membrane around it. If, on the other hand, it is kept in motion especially if it becomes broken and spread out over the general peritoneal surface it is not enclosed by peritoneum but is absorbed directly.

Whether the eggs are absorbed directly or after they are enclosed by peritoneum must depend partly on the rapidity with which they enter the body cavity, partly upon the strength

of peristaltic movements of the intestines and oviduct, together with the chance of being lodged in some undisturbed position, and partly also upon the general physiological tone of the individual and the consequent power of absorption from the general peritoneal surface.

From his study of "the effect of variations in body position and in gastrointestinal activity upon the degree and extent of diffusion of benign and toxic substances when introduced intraperitoneally" and of "the influence of the degree of diffusion upon the absorption of toxins" Yates ('08) concludes that:

1. Intraperitoneal diffusion results from the operation of purely physical forces abetted by physiological activities.

2. The greatest attainable restriction of this diffusion must result from the opposition of controllable physical force to the paths of greatest extension and the inhibition of physiological activities.

3. Under similar conditions the rate of absorption varies directly with the extent of diffusion.

Our observations on the absorption of eggs discharged into the body cavity are in general agreement with Yates' experimental results.

When a bird is laying she is transforming a large amount of material into egg substance. Gerhartz ('14) has recently made accurate measurements of the energy contained in the egg and of that utilized in the digestion and absorption of the extra food and in converting body substance into egg substance. With the laying of the egg all this energy is lost to the individual. When the egg is deposited in the body cavity and resorbed the energy contained in the egg, minus the energy required for the resorption, is retained by the individual. When egg formation is stopped at ovulation, the ovulated yolk being resorbed from the body cavity, not only is the energy contained in the yolk saved to the individual but also the energy which would have been contained in the egg envelopes and that which would have been utilized in their elaboration. If the energy consumed in the resorption of yolks or eggs from the body cavity is less than the energy contained in fully formed eggs,

and if birds which are ovulating or extruding eggs into the body cavity continue to digest and absorb the same amount of food as birds in similar periods of reproduction, the birds which are prevented from laying eggs will accumulate reserve energy.

The experiments so far as they go suggest that birds which resorb yolks or eggs from their body cavity may utilize this resorbed material (and the material not used to complete the eggs) in the production of body fat. There are six cases (1, 9, 10, 13, 14 and 15) which show evidence on this point. These birds were all killed for data while in good health except cases 1 and 13. Case 1 died as the result of the rupture of an enlarged spleen. Case 13 died from the infection of the body cavity through an abdominal hernia. All these birds had made large gains in body weight since the operation and all were *very fat* at the time of autopsy. The autopsy records quoted on page 412 show that the unoperated birds which were ovulating into the body cavity and resorbing the yolks were also very fat. The other birds which had ovulated into the body cavity were either killed too soon after the operation to have resorbed many yolks (cases 4 and 8) or died from diseases which might have prevented the accumulation of body fat (cases 7, 12, 16, 18, 19 and 26). Some of these cases also had made substantial gains in body weight but they did not appear to be abnormally fat at the time of autopsy.

Too much weight cannot be placed on the evidence from these experiments, since most of the birds were not fully grown at the time of operation and a part of the gain in body weight is due to normal growth. Further the cases are too few to determine statistically that these birds had accumulated more body fat than laying birds of the same age. This, however, appears to be the case.

THE EFFECT OF OPERATIONS ON THE OVIDUCT UPON ITS GROWTH
AND ABILITY TO FUNCTION*1. Diminishing the caliber of the duct*

Four operations were performed to test the effect of diminishing the diameter of the oviduct. In cases 21 and 22 the size of the albumen secreting region of the duct was decreased for several centimeters by taking a tuck in the wall. In case 23 the size of the shell gland was diminished by cutting out a piece of the wall. In case 24 the shell gland was made smaller by inverting a longitudinal portion of the wall. All these birds laid normal eggs. In size and shape these eggs were within the range of variation shown by the eggs of unoperated birds of the same breed. At autopsy the stitches which had made the tucks had either pulled out (cases 21 and 24) or the tuck was preserved only as a ridge on the muscular wall (case 22). In case 23 where a piece of the wall of the shell gland had been removed this part was of normal shape. It looked a little small. This bird, however, had laid eggs fully up to the average size for her breed.

The operations attempted did not perceptibly influence the size or shape of the egg. Many well controlled experiments would be necessary to demonstrate that it is impossible to alter the size and shape of the egg by diminishing the diameter of the tube. In three of the four operations the size of the tube was not permanently diminished because the stitches did not hold.

2. Closing the duct at various levels

When the funnel is closed by sewing the lips, or by ligating the *ostium*, yolks cannot enter the duct. Yet in such cases the duct passes through the normal phases of reproduction. In cases 1 to 6 the oviducts were in laying condition at the time the ostium was closed, either by sewing the lips (case 1) or ligating the funnel mouth (cases 2 to 6). At the time of autopsy thirteen days to two months later the oviduct of each bird was in a condition of reproduction corresponding to the condition of the ovary. Two of these birds had ovulated into the body

cavity but there is no evidence that any secretion had been formed by the duct. Closing the mouth of the duct does not prevent the duct from passing through the cyclic changes which are coördinated with the cyclic changes in the ovary. The duct glands, however, do not ordinarily discharge their secretion except under the stimulation of the presence of the yolk. This is in accord with the result of Pearl and Surface ('09 a) on the shell gland.

Cases 9 to 14 show the effect of closing the duct at other levels. The duct was closed by a ligature in cases 9 and 10; by an operation which resulted in the permanent shrinking of a small portion of the tube in cases 11 and 12; and by the section of the duct between two ligatures in cases 13 and 14. In all these cases the oviduct was in full functional condition at autopsy. In every case the duct on both sides of the ligature or cut had enlarged to the size of an oviduct in a laying bird. Yolks had entered these ducts and had stimulated the secretion of the duct as far as the point where the passage was closed. In no case was there any secretion posterior to the ligature. That is, the whole duct enlarged but it was able to function only under the stimulation of the advancing egg.

There is possibly a difference in the final response of the duct according to the level at which the passage is stopped. Where the duct was closed through the shell gland (cases 9 and 14), membrane covered or soft shelled eggs were formed and passed back up the duct into the body cavity. In cases 11 and 12 the ducts were closed at the isthmus and in each case the duct above this point was filled with masses of secretion which surrounded yolks but they did not pass back into the body cavity. In case 12 there were yolk masses in the body cavity surrounded by peritoneum but these did not appear to contain albumen. It is possible that an egg cannot stimulate sufficient antiperistalsis to cause its extrusion from the duct until it has become somewhat firm by the formation of the egg membrane. The number of cases, however, are too few, and the possibility of the operation of other causes too great, to place much weight on this interpretation.

3. Removal of the larger part of the duct

In cases 15 to 20 the oviduct was almost entirely removed. In most cases a portion of the funnel and a portion of the vagina were left. All these ducts were far from laying condition at the time of the operation. Case 17 was just beginning to enlarge for its first laying period. It was only 15 cm. long. The others were in infantile or non-laying condition. An examination of the autopsy records made from seven months to four years and eight months after the operation shows, first, that whatever portion had been left at the time of operation had remained, and second, that at the time of autopsy it exhibited the condition which one would expect to find for that portion of the entire oviduct in a bird with a similar condition of the ovary. This shows that the removal of the greater portion of the oviduct does not cause the atrophy of any remaining portion. In fact any part of the duct not removed apparently passes through growth and cyclic changes associated with the periods of ovarian yolk production exactly the same as though the duct were intact. This result was obtained also by Sellheim ('07).

4. Section or removal of all or a part of the ventral ligament

The peritoneal sheet which enfolds the oviduct projects as a fold along the ventral margin of the duct. In this fold of peritoneum there is developed a considerable amount of smooth muscle. This peritoneal fold with its contained muscle has been described by one of the authors (Curtis '10) as the ventral ligament of the oviduct. In a laying bird the extreme ventral margin of this ligament is a solid muscular cord, three to six millimeters in diameter. It becomes heavier toward its caudal end.

Muscle bundles pass from it to the duct where they are continuous with fibers in the longitudinal muscular layer of the wall. Other bundles pass to the colon and cloaca. Large bundles also cross the uterus and attach to the body wall muscles in the anal region. The forward end of the ligament suspends the backward prolongation of the funnel lips.

The anatomical relations of the ligaments and the distribution of the muscle fibers suggest that it aids in the peristaltic movements of the duct and may be of great importance both in the reception of the yolk by the duct and in the extrusion of the mature eggs. Cases 25 to 29 were planned to test the part played by the ventral ligament. In case 25 it was cut and in cases 26 to 29 all or a part of it was removed.

Case 25 was a laying bird with an egg in the shell gland at the time of operation. The round cord of muscle on the ventral side of the head of the shell gland was cut. On the next day the bird laid the egg which was in the shell gland at the time of operation. She never laid again. The anatomical effect of the operation is not known since the bird was accidentally sold more than a year after the operation. The operation did not prevent the laying of the egg already formed but apparently prevented the laying of any others.

Case 26 was a three months old pullet with the oviduct in infantile condition at the time of operation. The entire ventral ligament was removed. The bird never laid. At autopsy eight and one-fourth months after the operation it was found that she had ovulated into the body cavity. The oviduct was in the condition corresponding to the condition of the ovary but there was no sign that a yolk had ever entered the duct.

At the time of operation case 27 was a five and one-half months pullet with oviduct still in infantile condition. Three-fourths of a centimeter of the ventral ligament in the isthmus region was removed. The bird developed normally and laid several normal eggs. At autopsy four and three-fourths months after the operation the ventral ligament was found in normal condition at the upper end. The funnel attachments were normal. The ligament was lacking entirely in the isthmus region. On the ventral and lateral wall of the uterus closely adhered to the wall was a thick mass of muscle which extended across the uterus and sent fibers both dorsally and ventrally along the uterus wall. Also there was a bundle of fibers which passed along the ventral margin of the wall and on to the vagina. The distribution of these fiber bundles was quite different from

the distribution of those of a normal ventral ligament but their contraction would be very effectual in diminishing the size of the uterus and therefore in expelling the egg.

Cases 28 and 29 were also young pullets from which portions of the ventral ligament were removed before the first enlargement of the reproductive apparatus. Neither of these birds ever laid. They were autopsied when in strictly non-laying condition. The ventral ligament was lacking at the head of the uterus. If they had ever passed through periods of reproductive activity in which they ovulated into the body cavity, they had completely resorbed all the yolks. These cases do not therefore contribute to our knowledge of the function of the ventral ligament since there was no proof that either bird had been in laying condition. They, however, do agree with the results from the other cases that if any portion of the ligament is removed it is not replaced but all the remaining portions develop. In case 27 described in the preceding paragraph there was, however, a compensating development of muscle fibers in the uterus wall so that expulsion of the egg was possible.

These experiments on the ventral ligament point strongly to the conclusion that the action of the forward part of the ligament is of great importance for the ovulation of the yolk into the duct either by holding the funnel in position or by the movements induced in the funnel. Since an egg cannot be extruded unless it is formed, only two of these cases (25 and 27) have any direct bearing on the question of the function of the ligament in egg laying. Each of these birds could extrude eggs after the operation on the ligament. In case 25 the ligament was simply cut across. It is quite possible that this may not have greatly interfered with the normal muscular action of the muscle bundles in the uterus region. In case 27 there was an unusual development of the muscle fibers on the wall of the uterus. The contraction of these fibers would have much the same effect as the contraction of the muscle bundles from the ligament. This suggests that the muscle bundles contained in and arising from the ventral ligaments are probably an important part of the normal apparatus which expels the egg.

SUMMARY

1. Neither the ligation, section, nor entire removal of the oviduct causes the degeneration or prevents the further growth of the ovary.

2. The pressure of the enclosing funnel is evidently not necessary to ovulation since yolks are ovulated into the body cavity after the *ostium* is sewed or ligated or after the entire duct is removed.

3. Internal pressure due to continued yolk formation is probably the most important factor in the normal rupture of the follicle, since closing the funnel or removing the duct apparently does not greatly delay ovulation.

4. There are cases of unoperated birds with normally functioning ovaries, and oviducts apparently capable of functioning, which do not produce eggs because of some anatomical or physiological condition of the mouth of the oviduct which prevents the entrance of the yolk.

5. The fate of yolks or eggs set free in the body cavity depends apparently upon the physiological vigor of the bird. First, they may cause serious metabolic disturbances which result in the death of the bird; second, they may be absorbed rapidly from the general peritoneal surface; or third, they may be walled off by peritoneum and then absorbed.

6. The material from the resorbed yolks or eggs is apparently utilized in body metabolism since all such birds which were in good health at the time of autopsy were very fat.

7. The removal of the greater portion of an oviduct does not cause the atrophy of any remaining portion.

8. The whole or any remaining part of an oviduct sewed at the funnel, ligated at any level, or with parts removed, passes through growth and cyclic changes coördinated with changes in the ovary exactly as an unoperated duct.

9. The stimulation of the advancing egg is necessary for the discharge of the secretion of the duct, since a duct closed at any level functions only to the point where the passage is interrupted.

10. When any portion of the ventral ligament is removed it is not replaced but all remaining portions develop.

11. The forward portion of the ventral ligament is necessary for the reception of the yolk by the funnel.

12. The muscle bundles which arise from the muscular cord in the ventral ligament along the uterus are probably an important part of the normal apparatus which expels the egg.

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A NORMAL PERIODIC REORGANIZATION PROCESS WITHOUT CELL FUSION IN PARAMAECIUM

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SIXTY-SIX FIGURES (FOUR DOUBLE COLORED PLATES)

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I. INTRODUCTION

The conception of the Protozoa as primitive animals has led to innumerable studies on these forms—morphologists and physiologists alike being animated with the idea that here many of the riddles of life propounded by the so-called higher animals would be presented in a simple form and so be more readily solved. While this view of the Protozoa is undoubtedly true

in a broad sense, recent studies on these organisms have only served to emphasize that size and simplicity do not necessarily go hand in hand and that a unicellular or non-cellular animal with all its varied life processes performed within the confines of a protoplasmic unit presents difficulties which differ in kind rather than in degree from those encountered in the metazoa. No better instance of the truth of this could be presented than that afforded by studies on the life history of the Infusoria. Dujardin's ideas of simplicity—brought forward to combat Ehrenberg's mistaken interpretations of the complexities which he observed—gradually were displaced by the revelations of Balbiani, Bütschli, Engelmann, Maupas, Hertwig and others on the complicated phenomena of conjugation, while the results of more recent work on conjugation from the standpoint of dynamics and heredity have only served to emphasize the intricacies of the infusorian life processes. The present study fully describes still another complex nuclear phenomenon¹ which we have discovered in the life of *Paramecium aurelia* and which we believe affords the key to certain apparent contradictions in recent results on the life history of the Infusoria.

The problems of protoplasmic senescence and the significance of fertilization have afforded the stimulus for a long series of investigations on the life history of Infusoria since, more than three-quarters of a century ago, the leading students of microscopic organisms, Ehrenberg and Dujardin, theorized on the potential immortality of these forms. It remained, however, for Bütschli ('76) and Engelmann ('76) to attack the problem experimentally, in the light of zoological advance in the intervening years, and to reach the conclusion that continued reproduction by division results in degeneration and death; while the classic studies by Maupas ('89) on the life history of a number of different species of Infusoria afforded such a wealth of evidence pointing in the same direction that conjugation as a sine

¹ We have already given brief outlines of this process in the *Proc. of the Society for Exper. Biol. and Med.*, vol. 11, no. 3, February 18, 1914, and in *Biol. Cent.*, Bd. 34, 1914. In the latter several errors are present owing to the war preventing a revision of proof.

qua non for the life of the Infusoria seemed to be placed upon a firm empirical basis.

A series of careful investigations by R. Hertwig ('00-'04) and Calkins ('02-'04) confirmed Maupas' general conclusion that Infusoria after a more or less definite number of divisions degenerate and finally die if conjugation is prevented. Further, the latter author found that artificial stimuli of different kinds may be substituted with success for conjugation since by the opportune use of artificial stimulation he was able to prolong the life of one culture of *Paramecium caudatum* to the 742d generation. This significant discovery that the death of infusorian cultures, bred on a more or less constant medium of hay infusion, may be temporarily deferred by artificial means was corroborated by Woodruff ('05).

Enriques ('03) studied the same general problem and reached the conclusion that the degeneration and death observed by Calkins and others was due to the presence of bacterial poisons in view of the fact that he succeeded in breeding *Glaucoma scintillans* for 683 generations without signs of degeneration when this factor was eliminated. Although Enriques' interpretation of the cause of degeneration in Calkins' cultures is open to question, the significant fact remains that he kept his animals nearly twice as long as did Maupas or Calkins without conjugation or artificial stimulation, thus suggesting that degeneration is not inevitable, and that if suitable conditions are supplied reproduction by division can proceed indefinitely.

The problem was attacked from another point of view by Woodruff (1907 to date) who investigated the possibility that the degeneration observed in the previous investigations was induced by too great uniformity in the conditions of culture, or by the culture medium being deficient in something essential for the continued well-being of the organisms. A race of *Paramecium aurelia* (I) was isolated and bred on infusions of various materials found in the natural environment of the animal, while a sub-culture was subjected to the relatively constant hay infusion culture conditions employed by Calkins ('04). The result was that the cells bred in the constant hay infusion medium died

out after a typical Calkins cycle, while those bred on the 'varied environment' medium did not pass through periods of marked physiological depression or show morphological changes which could be interpreted as abnormal. This race is still (June, 1914) in a normal condition, having attained over 4500 generations without conjugation or the use of artificial stimuli.

The success with the varied culture medium naturally led to the question whether the longevity of *Paramecium* on a varied environment is dependent upon intrinsic stimuli from the frequent changes of the medium, or whether a constant medium of hay infusion is unfavorable because it lacks some elements which are essential for the continued existence of the organism. Accordingly, Woodruff and Baitzell ('11 a) bred a sub-culture of this race for a period of nine months on a constant culture medium of beef extract. The continued health of the organisms on this constant medium throughout the experiment, which was continued sufficiently long to include a Calkins cycle if such was inherent, indicated that it is the composition of the medium rather than the changes in the medium which is conducive to the unlimited development of this race without the necessity of conjugation or artificial stimulation.

From a study of various species of hypotrichous Infusoria, as well as his main culture of *Paramecium aurelia*, Woodruff found that minor periodic rises and falls of the division rate occur from which recovery is autonomous. He termed these fluctuations 'rhythms' and contrasted them with the so-called cycle, which comprises a varying number of rhythms and, according to Maupas and Calkins, ends in the death of the race if conjugation or artificial stimulation is not resorted to. The problem of the rhythms was studied intensively by Woodruff and Baitzell ('11 b) who showed that when *Paramecium aurelia* is subjected to the most constant environmental conditions it is impossible to eliminate the rhythm and thus resolve the graph of the multiplication rate into an approximately straight line. Accordingly there are inherent rhythmical changes in the phenomena of the cell which produce slight changes of the division rate. The same result was reached by Woodruff and Baitzell

('11 c) from a study of the temperature coefficient of the rate of reproduction of this culture which showed that the rate of cell division of *Paramecium* is influenced by the temperature at a velocity similar to that for a chemical reaction—except when the rhythms interfere.

The results from the study of this pedigreed race of *Paramecium aurelia* have led Woodruff to conclude that this organism, when subjected to suitable culture conditions, has the power of unlimited reproduction by division without conjugation or artificial stimulation—the only necessary variations in the rate of reproduction being the normal minor periodic rise and fall of the division rate (rhythm), due to some unknown factor in cell phenomena, from which recovery is autonomous.

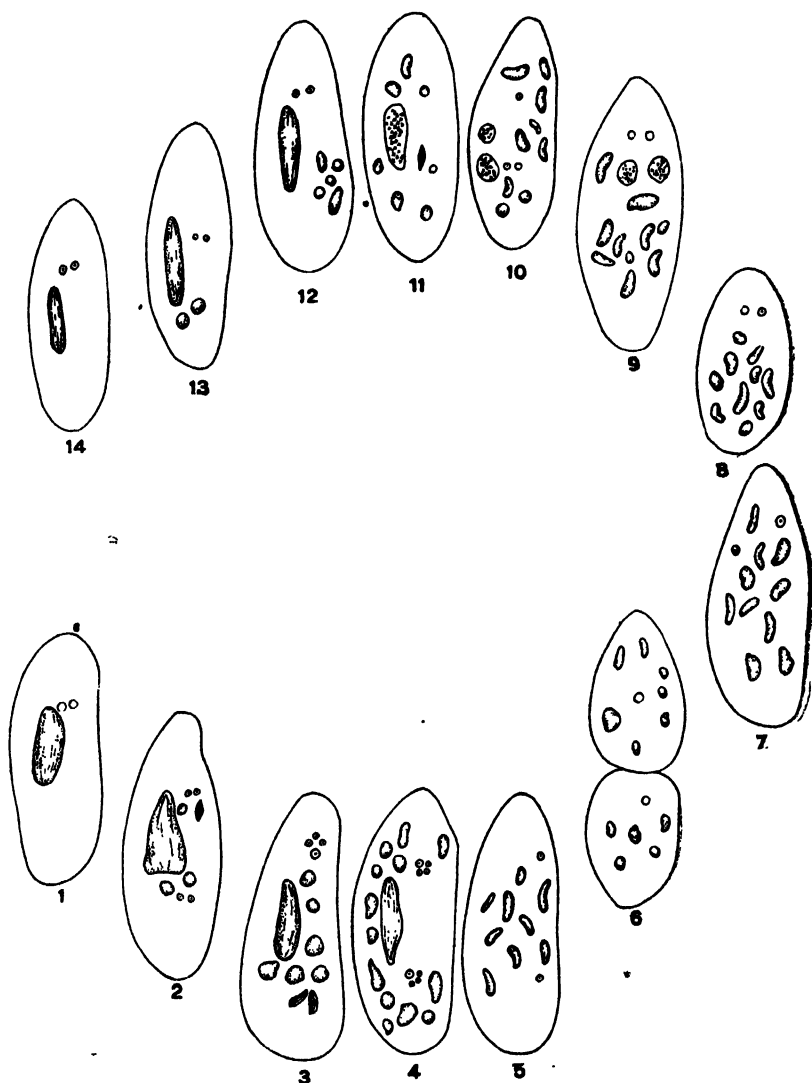
Calkins and Gregory ('13, p. 507) “ . . . do not share his optimism, however, and can only say that while his results are remarkable, his race is not yet dead. Is there any clue to the particular make up of this race of *Paramecium aurelia*?” Accordingly Calkins sought the explanation of the diametrically opposite results derived from his and from Woodruff's cultures of *Paramecium* in variations in the tendency to conjugate which have been observed by Jennings ('10) and himself to exist in different races of this organism. Thus he emphasized the fact that he could readily induce conjugation in his culture whereas experiments to secure conjugation in Woodruff's culture were without effect. Calkins, therefore, stated that “the two races cannot be compared in regard to vitality, since normal conjugation was prevented in the conjugating race, whereas in the non-conjugating race there has been no artificial prevention of a normal process.” “Woodruff's *Paramecium aurelia* is evidently a *Paramecium Methuselah* belonging to a non-conjugating line the life history of which is not known in any case.”

However, conjugation was finally secured in a mass culture seeded from Woodruff's race ('14) thus demonstrating that this race is a conjugating race when the proper conditions for its consummation are realized. Therefore, there is no evidence extant that a non-conjugating race of *Paramecium* exists.

With this theory swept away by a fact the results derived from this culture stand where they stood before and demonstrate that the very limited periods in which Maupas, Calkins, and others observed degeneration have no significance for the question as to whether degeneration and death are inevitable results of reproduction without conjugation. In other words, this one positive result from Woodruff's race outweighs all the negative evidence derived from work on the Infusoria, and justifies the statement that these organisms can live indefinitely, when subjected to favorable environmental conditions, without conjugation or artificial stimulation.

Although morphological or physiological variations which could be interpreted as the result of degeneration were never observed in Woodruff's race of *Paramecium*, it was early noted in culture "that various nuclear changes which are not at present organized occur normally in the life history of *Paramecium*," and suggested that possibly when conjugation is prevented, a reorganization of the nuclear apparatus within the individual cell occurs. Erdmann ('08) independently reached an essentially similar view from a consideration of the published data on this culture and a critical study of infusorian life histories, and further, in an experimental study of *Amoeba diploidea* ('13), suggested that a relation exists between sexual phenomena and rhythms. Accordingly the present authors have collaborated in a study of the daily cytological changes of this race of *Paramecium aurelia* (I).

As a result of our study of a large series of animals preserved daily during the past half year and of specimens preserved at various periods throughout the existence of this culture of *Paramecium*, we have discovered that the rhythms in the division rate are the physiological expression of internal phenomena which involve the formation of a complete new nuclear apparatus, by a definite sequence of normal morphological changes which simulate conjugation. This nuclear reorganization, in essence, consists of a gradual disintegration and absorption of the macronucleus in the cytoplasm. Simultaneously a multiplication of the micronuclei is in progress. Certain of the resulting micronuclei degenerate while the remaining one or two form the new



Text fig. 1 Diagrammatic survey of the reorganization process. Descending phase, 1-5; climax, 6-9; ascending phase, 10-14. Dot in circle = micronucleus; isolated dot = degenerating micronucleus; lines and dots = macronucleus; stipple = chromatin body; crosses = macronuclear anlage.

macronuclear and micronuclear apparatus. This results in the reorganization of the cell without the fusion of two animals (text fig. 1).

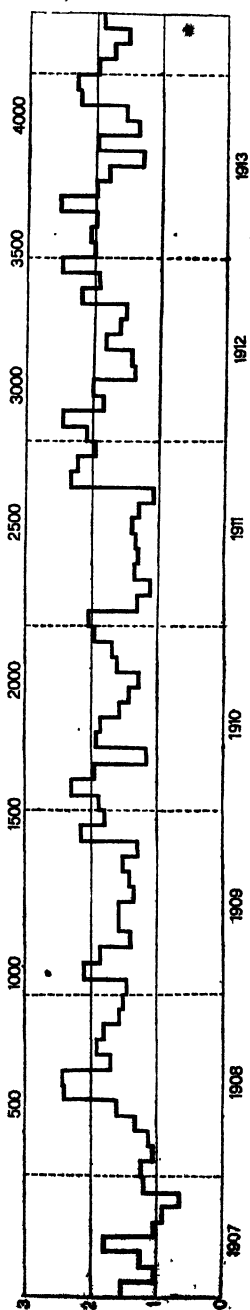
II. MATERIAL AND METHODS

A. METHODS OF CONDUCTION OF THE MAIN CULTURE I

Since the results presented in the present paper are based on a cytological study of the main pedigreed race (I) which has been carried on for the past seven years, a brief outline of the methods employed in its conduction must be given. For further details the reader is referred to earlier papers on this race (Woodruff '08, '11).

A specimen of *Paramaecium aurelia* was isolated from a laboratory aquarium on May 1, 1907, and placed in about five drops of hay infusion on an ordinary glass slide having a central depression. When the animal had divided twice, producing four individuals, each of these was isolated in fresh media on a separate slide, thus forming the four main lines (Ia, Ib, Ic, Id) of this culture, *Paramaecium aurelia* I. This pedigreed race has been maintained by the isolation of a specimen from each of the four lines practically every day during the more than seven years of its existence. The isolations have been made with a capillary pipet and a Zeiss binocular microscope, oculars 2, objectives 55a. This daily isolation has prevented the possibility of conjugation occurring, afforded fresh culture medium, and enabled an accurate record of the generations to be kept. Therefore this is a pedigreed culture of *Paramaecium*. The rate of division of the culture has been determined by averaging the daily rate of these four lines and the accompanying graphs show this again averaged for various periods as indicated in the respective legends (text fig. 2).

The culture material supplied to the main lines of the race has consisted of thoroughly boiled infusions of materials taken practically at random from ponds, swamps, etc., in an endeavor to supply the general type of material ordinarily met with by this organism in nature. The depression slides holding the animals have been kept in a glass moist chamber on a laboratory table and therefore the organisms have been subjected to the ordinary fluctuations of temperature, light, etc., of the room. The morphological condition of the animals has been followed



Text fig. 2 Graph showing the rate of reproduction of the pedigreed race (I) of *Paramaecium aurelia* from start on May 1, 1907, to May 1, 1914, at the 4594th generation. The ordinates represent the average daily rate of division of the four lines of the culture again averaged for each month of its life to date. The vertical broken lines indicate the limits of the calendar years. The figures 500, 1000, etc., represent generations and are placed above the periods in which they were attained. (*The fluctuations in the curve are chiefly the result of the varied culture conditions and therefore must not be confused with rhythms.*)

in a general way by periodically preserving specimens left over at the daily isolations.

The culture was started and conducted for two months at the Biological Laboratory of Williams College, and since has been carried on at the Biological Laboratories of Yale University, except during a part of each summer when it was transferred to the Marine Biological Laboratory at Woods Hole, Mass.

It is under these conditions then that this culture (I) has been successfully maintained without loss of vigor for over 4500 generations—all the experiments which have been conducted on animals from this race having been made on sub-cultures started from the animals left over from the lines of this culture at the daily isolations.

B. METHOD OF CONDUCTION OF SUB-CULTURE IE

For the intensive study of the daily cytological changes of this race of *Paramecium aurelia*, on October 27, 1913, six animals left over from Line Ia of the main culture at the 4020th generation were isolated to start a new sub-culture, designated IE, of six lines. This sub-culture was subjected to practically constant environmental conditions. The culture medium was the 0.025 per cent beef extract which Woodruff and Baitzell ('11 a) found to be a most favorable medium for this race of *Paramecium*. The sub-culture was kept in a thermostat set for 26°C., and such variations (about 1°C.) from this as occurred were well within the optimum zone for the animals of this race as determined by Woodruff and Baitzell ('11 c).

An animal was isolated every day from each line of this sub-culture from its initiation to April 27, 1914—a period of six months. The isolated animal from each line was placed in fresh culture medium, while one or more of the remaining animals was preserved for study. Thus for half-a-year permanent preparations were made from day to day of sister cells, the exact ancestry of which was known in every case. *It is important to emphasize the fact that by this method only, i.e., the study of practically each cell generation, could the sequence of nuclear changes which we describe be determined.* This became evident very soon as the

work proceeded and consequently the study was concentrated particularly on one (VI) of the six lines—the other lines being merely controlled to show the occurrence of the process, at which time all the cells available from these lines were also preserved. In many cases all the animals of certain lines were killed in the reorganization process so that every animal could be studied. The places of these lines were supplied by new ones from one of the remaining five lines. Thus by killing of various lines and the branching of others to take their places a total of fifteen sub-lines of longer or shorter duration were formed. These together with the six original lines made a total of twenty-one lines whose cytology was investigated.²

The individual animals were fixed in Schaudinn's sublimate-alcohol (stronger solution), stained with Delafield's hematoxylin and mounted in cedar oil—the specimen being watched under a Zeiss binocular microscope throughout the operations, and transferred with a capillary pipet from one depression slide to another as occasion demanded. Differentiation was effected with acidulated alcohol (70 per cent alcohol + 0.002 per cent HCl) under a compound microscope. The following staining reagents were tried but the above methods gave the best results—bearing in mind that each animal had to be carried along under constant observation through each of the fluids until it was in the cedar oil under a coverglass: Heidenhain's hematoxylin and Bordeaux red, methyl green and eosin; Delafield's hematoxylin and eosin; safranin and methyl green; Mannsche Färbung and Giemsa feucht. Animals preserved in bulk from mass cultures were submitted to the same staining methods and again Delafield and eosin were found most satisfactory; Heidenhain staining the trichocysts too greatly. Sections (5μ) were made of animals in various stages of the process but they did not afford important details which could not be made out in the total mounts. The sections were stained with safranin and Heidenhain according to the suggestions of Enriques ('12).

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² After the definitive experiments were formally concluded at the end of six months, certain lines were continued to June 14, 1914, in order to secure some further details.

In addition to this material preserved daily from Sub-culture IE, we had at our disposal, as already mentioned, preparations preserved at various periods throughout the life of the main culture (I). These had been fixed in a saturated solution of corrosive sublimate with 5 per cent glacial acetic acid, stained with Ranvier's picrocarmine and mounted in damar.

III. DESCRIPTION OF THE CYTOLOGICAL CHANGES IN THE REORGANIZATION PROCESS

The general outline of this remarkable cytological process which accompanies the rhythms clearly shows that a complete internal reorganization of the *Paramecium aurelia* cell occurs without cell fusion. The details to be presented were obtained as already described from Sub-culture IE, from October 27, 1913, to April 27, 1914, and certain stages were substantiated with specimens which had been preserved from the main culture (I) at various isolated periods during the previous six and one-half years of its existence. Figure 1 (pl. 1) shows a typical specimen of *Paramecium aurelia* isolated from the main culture (I) on April 11, 1908, at the 424th generation, i.e., 3596 generations before Sub-culture IE was started. Figure 2 (pl. 1) represents an animal in the 4020th generation at the time Sub-culture IE was branched from the main Culture I.

The reorganization process resolves itself naturally into three periods: the descending phase, the climax, and the ascending phase.

A. DESCENDING PHASE

The macronucleus of a typical *Paramecium aurelia*, which is not undergoing the reorganization process, consists of fine chromatic granules enclosed within a relatively thick membrane. The finer structure has been fully described by Maupas ('89, p. 217) and Hertwig ('89, p. 9). The two micronuclei, in a similar period with respect to the process and in the resting stage between two cell divisions, are more or less compact and homogeneous, and often lying in a pair near the macronucleus which sometimes obscures them from view. The cytoplasm of the cell

contains many small vacuoles some of which may be filled with bacteria (figs. 1 and 3, pl. 1).

As the low point of the division rate approaches, the granules of the macronucleus become more and more coarse, its staining capacity increases, its form becomes more kidney-shaped and its shorter axis elongates. Still more obvious changes of the macronucleus indicate the actual beginning of the regulation process.

1. *The macronucleus*

At this stage projections appear at the end of the macronucleus (figs. 4 and 5, pl. 1) which are either merely thin membranes devoid of chromatin or filled with small granules. Hertwig ('89, p. 7) describes them as follows: "gleichzeitig verliert die Oberfläche ihr glattes Aussehen; Einkerbungen erstrecken sich mehr oder minder tief in das Innere hinein und zerlegen den Kern nicht selten in drei ungleich grosse Lappen oder es werden an den Enden fingerförmige Fortsätze deutlich oder leisten- und riffartige Vorsprünge." According to this author they are not evident during the latest stages of vegetative cell division, or in the first changes incident to conjugation. Though Hertwig could not observe these projections in the living animal he did not believe them to be artifacts because he could demonstrate them with every fixation fluid. We have never observed them in vegetative cell divisions but frequently have noted their presence several generations before the definite onset of the process. We have no data in regard to their relation to conjugation.

Figure 5 (pl. 1) gives a good idea of these projections. The animal figured is from Line VI, 4094th generation, and is nine generations before the beginning of the reorganization process which started in the 4103d generation. These long finger-like projections are preceded by the appearance of smaller ones (fig. 4, pl. 1) which seem to indicate that plasmatc currents are present in the macronucleus though the membrane at this stage is still intact.

The next stage which is peculiar to the process is the separation of chromatin bodies from the macronucleus. In the early stages of the reorganization process cells are seen with only two or more chromatin bodies (figs. 6 and 7, pl. 1) which are more or less spherical and consist of large and small granules. Figure 8 (pl. 1) shows a macronucleus which is surrounded by several of these bodies which have been ejected from it. This method of nuclear disintegration is not unique in Infusoria because similar morphological phenomena are described by Neresheimer and Buschkiel. Neresheimer ('08) mentions that chromatin bodies are ejected from the macronucleus of *Ichthyophthirius* before the beginning of the sexual process. This species during vegetative life has its macronucleus and micronucleus in one body, but this difference is unimportant because the chromatin bodies of *Ichthyophthirius* contain the combined material of the macronucleus and micronucleus, and at the time of extrusion the vegetative or sexual character of the chromatin bodies is determined. In *Paramaecium aurelia* merely vegetative nuclear material is extruded by the macronucleus.

A connection between the macronucleus and the chromatin bodies of *Paramaecium* as described by Buschkiel in *Ichthyophthirius* ('08, p. 81) could not be discovered and seems not to exist because these bodies are formed in the macronucleus and are ejected through openings in the membrane. The formation of the chromatin bodies begins in the macronucleus and is evident by the condensation of granular material at various points within the membrane (fig. 8, pl. 1), other parts of the macronucleus becoming devoid of chromatin as segregation proceeds. The chromatin bodies are not yet surrounded by clear areas. The resulting membranous condition of the 'old macronucleus is shown in figures 6, 10 and 11 (pl. 1) and figure 14 (pl. 2). Two characteristic stages in the elimination of chromatin bodies by the macronucleus are given in figures 8 and 9 (pl. 1).

The chromatin bodies are removed from their place of origin by movements of the cytoplasm, thus emphasizing the well-known cytoplasmic currents in the cell. The specimen shown

in figure 10 (pl. 1) has seven of these bodies arranged in a semi-circle at the posterior end of the cell. The anterior end contains some irregularly shaped chromatin bodies which seemed to be undergoing involution, while three others more recently ejected are still near the macronucleus. The disintegration of the macronucleus progresses until it is entirely devoid of chromatin (figs. 35, 36, pl. 4) while the wrinkled and ruptured membrane with but slight staining capacity remains in the cell (fig. 14, pl. 2.) In the later period of the reorganization process the membrane is resorbed and the old macronucleus has finished its function in the paramaecium cell.

These changes of the macronucleus have their analogy in normal conjugation. There the old macronucleus is destroyed but this is effected by the formation of the so-called "wurstförmige Schlingen," which form a tangled mass of chromatin ribbons. Their origin is figured by Maupas (fig. 10, pl. 12; figs. 17-20, pl. 13); and by Hertwig (figs. 6-9, pl. 1; figs. 1-9, pl. 2) and in the present paper (fig. 44, pl. 4). However, in conjugation it is not until after the animals have separated that the chromatin ribbons are totally fragmented and more or less spherical bodies are free in the cell. Later an involution of these occurs, the details of which are not described by either Hertwig or Maupas. Likewise, the persistence of the membrane of the macronucleus is not figured by these authors. This ribbon-like formation of the macronucleus we have found to be characteristic of conjugating animals from this race of *Paramaecium aurelia* (fig. 44, pl. 4). Figure 32 (pl. 3) shows a pair of conjugants with their macronuclei in this form.

In this period then the differences between the macronuclear changes during conjugation and during the process are only morphological; on the one hand, the macronucleus forms 'wurstförmige Schlingen;' on the other the macronucleus eliminates its chromatin by extruding it in the form of spherical bodies. The physiological effect is the same. The result is the destruction of the old macronucleus.

2. *The micronucleus*

While these changes are taking place in the macronucleus the micronuclei do not remain unaltered. They move from their accustomed position, which is more or less close to the macronucleus, and migrate in the cytoplasm (fig. 3, pl. 1). The same appearance was noted in *non-conjugating* *Paramecium caudatum* by Calkins and Cull ('07, p. 383), who state that in stages of a pedigreed race, which Calkins calls depression periods, the micronucleus migrates in the cytoplasm, and they interpret this as an abnormal condition. In *conjugating* animals the migration of the micronuclei at the beginning of the sexual process is described in *Paramecium aurelia* by Maupas ('89, p. 212) and by Hertwig ('89, p. 21); in *Paramecium caudatum* by Calkins and Cull ('07, p. 383); and in *Paramecium bursaria* by Hamburger ('04, p. 200). This migration seems to be a general phenomenon at the onset of the changes incident to conjugation and occurs also, as described above, in isolated *paramecia* before the beginning of the reorganization process.

The micronuclei in their new position proceed to divide with the result that finally eight are present in the cell. Figure 14 (pl. 2) shows a specimen in which there are two solitary micronuclei and two groups of three micronuclei. All the micronuclei show a perfectly normal structure but some variation in size is evident. In typical conjugating animals the eight so-called reduction micronuclei are described by Hertwig and Maupas as all being of the same size, but Maupas did not lay great stress on cytological details so that only Hertwig's statement is of weight. It is probable that the differences of volume shown by micronuclei in the process are due to variations in the amount of nuclear sap preliminary to the cessation of their functional activity.

It is impossible to determine whether these micronuclei of the animals in the process are actually reduction micronuclei because, here, as in the conjugation of *Paramecium aurelia*, it is not possible to count the number of chromosomes.

The formation of the eight micronuclei does not begin until the disintegration of the macronucleus is about finished. Figures 36 and 37 (pl. 4) illustrate the coincidence of the end of the macronuclear disintegration and the beginning of the multiplication of the micronuclei. The animals in figures 35 and 36 show the typical form of the macronuclear destruction which takes place in the reorganization process without the formation of 'wurstförmige Schlingen'. A slight resemblance to the characteristic macronuclear condition during conjugation is given in the isolated paramaecium of the 4087th generation, thus indicating that the macronuclear destruction in conjugation and in the reorganization process may sometimes show similar features. The animals shown in figures 36 and 37 possess either three or four 'reduction' micronuclei. The identification of these as 'reduction' micronuclei is based upon their position in a homogeneous protoplasmic layer, and the absence of a micronuclear membrane, together with the general morphological structure of the cell and its *fate*.

The diagnostic characters of the 'reduction' micronuclei are still more prominent in later stages. Figure 11 (pl. 1) shows a macronucleus partly devoid of chromatin, eight chromatin bodies and three micronuclei. Two of the micronuclei are apparently starting to divide. The anterior micronucleus seems to be in the process of forming another by an unequal distribution of the chromatin inside the membrane. This is the only micronuclear change of this type which we have observed and may well be due to some irregularity. The formation of mitotic spindles with long distinct threads and crescents have not been seen, and apparently exactly the same type of mitosis as described by Hertwig does not occur in the reorganization process. However, figure 31 (pl. 3) illustrates an animal, from a sub-culture of this race in which conjugation was allowed to occur, having division spindles which approximate to a certain degree some of the spindles figured by Hertwig. It is well known that the morphology of the mitotic apparatus varies at different phases of cell life, as for example was found to be the case in *Amoeba diploidea* by Erdmann ('11, p. 336).

Calkins and Cull ('07, p. 383) note that, during periods which they call depressions, the typical structure of the micronucleus of *Paramecium caudatum* is lost and it becomes abnormally large with the chromatin in a loosely granular condition. It is a remarkable coincidence that this occurs also in *Paramecium aurelia* in the descending phase of the process.

Figure 12 (pl. 1) shows the macronucleus, which now has become smaller, together with fourteen chromatin bodies free in the cytoplasm, and two just leaving the macronucleus. The cell possesses three micronuclei: two, lying near each other in a cytoplasmic layer free from granules, are in early division stages, while the third has nearly finished division. Thus the cell soon would have contained six micronuclei. Though four micronuclei are formed in normal vegetative division the beginning of the 'reduction' division is distinguishable from that of vegetative division because the four vegetative micronuclei are usually either lying in closely associated or widely separated pairs in the cell (fig. 7, pl. 1) while the 'reduction' micronuclei remain clustered together in a layer of homogeneous cytoplasm. Later stages showing seven and eight micronuclei are given in figure 13, pl. 1, and figure 14, pl. 2, already described. Thus at the end of the descending period of the reorganization process the *paramecium* cell possesses eight micronuclei, a shrunken membrane of the old macronucleus, and numerous chromatin bodies which have passed out from the macronucleus (fig. 14, pl. 2).

The important multiplication of micronuclei is not without precedent in non-conjugating animals. Maupas mentions a non-dividing animal with three micronuclei. Popoff ('09, p. 30) describes a specimen of *Paramecium caudatum* with two micronuclei which are just preparing to divide and form four without cell division ensuing. Also Kasanzeff and Calkins note the multiplication of the micronuclei in *Paramecium caudatum* without cell division. Popoff states further that in *Stylonychia mytilus*, after treatment with a medium with CO_2 in solution, the normal four micronuclei (p. 15) multiply until eight may be present. It is unnecessary at this point to discuss the theoretical

bearings of these cases (cf. p. 483) but merely to emphasize the fact that the multiplication of the micronuclei in an isolated cell is *not* an infrequent event, and that the fate of the cell alone gives the significance of the phenomena.

B. CLIMAX

The period of the reorganization process, designated the climax, is the most important of the three phases. Morphologically defined it extends from the total disintegration of the old macronucleus to the formation of the new macronuclear anlagen. Physiologically speaking it represents a stage in which the vegetative functions of the cell are relatively in abeyance and only the potentialities of the micronuclei are in evidence. At the end of the descending phase the paramaecium cell possesses eight micronuclei, twenty to thirty spherical chromatin bodies and the membranous remains of the old macronucleus (fig. 14, pl. 2). Figure 13 (pl. 1) gives an animal just as the degeneration of the micronuclei sets in. It shows six micronuclei, one of which is dividing, scattered in the cell, and numerous chromatin bodies but no trace either of the old macronucleus or of the macronuclear anlagen. Four micronuclei are closer together than the other three, indicating that the two groups arise from each of the two original vegetative micronuclei. The scattered position of the micronuclei indicates the beginning of their degeneration (see fig. 44, pl. 4) which shows the same feature in a conjugating animal.

The next change consists in the practically complete disappearance of the old macronuclear membrane and in the degeneration of the so-called reduction micronuclei to the number two (or one). It was not possible to trace the method of degeneration of the micronuclei, but it is positive that in the generation which follows the above described stage only one or two are present. Maupas and Hertwig figured the degenerating micronuclei in *conjugating* animals as homogeneous minute dots the structure of which is not discernible (Hertwig '89, fig. 7, pl. 1; Maupas '89, fig. 13, pl. 12). Hertwig at first could not determine

whether one or two of the reduction micronuclei remained and formed immediately without a further division the stationary and migratory nucleus, but in his final publication ('89, p. 31) he states definitely that seven reduction micronuclei degenerate but his *figures* do not absolutely prove this point (fig. 6, pl. 1; fig. 24, pl. 4). A micronucleus remains which is the source of the new micronuclear and macronuclear apparatus. Maupas ('89, p. 219) stated earlier than Hertwig that "Sept d'entre eux vont, en effet, passer à l'état de corpuscles de rebut et disparaître en se résorbant. Un seul persistera et continuera l'évolution fécondatrice," but as already mentioned, he does not give cytological details so that a perfect description of the degeneration of the micronuclei in *Paramecium aurelia* does not exist. It is easier to trace the degenerating micronuclei in *conjugating* animals because there the ribbon-like formation of the macronucleus is still intact (fig. 44, pl. 4). Many chromatin bodies are not free in the cell and consequently cannot be mistaken for degenerating micronuclei. Thus each tiny homogeneous granule in the cytoplasm can be identified as a micronuclear remnant, whereas in the reorganization process unconnected chromatin bodies are scattered freely in the cell and minute homogeneous granules intermingled with them may or may not be identified as degenerating micronuclei. However, certain bodies which have been observed we are inclined to interpret as micronuclear remnants. These will be indicated in the description of the plates.

The reorganization process proceeds in two different ways, as shown by the animals which were preserved at the climax, depending upon whether or not a *cell division* takes place at this period. The usual method is for a cell division to occur. In the cell which possesses no macronucleus the chromatin bodies change their shape, becoming more and more elliptical while each granule begins to migrate to the surface so that the bodies appear hollow. The one remaining micronucleus is large. Figure 15 (pl. 2) shows such cells which have just completed the division at the climax. Each has one micronucleus while the anterior cell has eight and the posterior twelve chromatin bodies.

The posterior animal still contains a piece of the old macronuclear membrane. It is possible that the two micronuclei in this dividing animal (fig. 15, pl. 2) are the result of a division of a *single* 'reduction' micronucleus which alone remained in the parent cell. It is also possible that *both* of these micronuclei are the 'reduction' micronuclei which did not degenerate and which are now, after a long period of passivity, distributed to each of the daughter cells. In this case the formation of a new spindle could not have occurred and the absence of the micronuclear division would be proof that the process is effected in this way. In eight cases of the process the cytological study has shown that a *cell* division actually does occur. Such cases fall under the heading Ia and Ib in text figure 3.

The discussion of the micronuclear events preceding the *cell* division in the climax will be postponed until all the cytological data from the study of the climax have been presented.

The morphology of the cell *after* the cell division in the climax was definitely determined from several specimens (see fig. 15, pl. 2, and figs. 41 and 40, pl. 4). Clearly the reorganization of the nuclear apparatus is effected by a single micronucleus in all cases in which a cell division occurs in the climax. Figures 41 and 42 (pl. 4) give two cells in the 4436th and 4437th generations respectively. Cell 4436 has one micronucleus and numerous chromatin bodies and is an animal *after* the cell division in the climax. Cell 4437, which is a product of the first division of the sister cell of cell 4436 after about forty-eight hours, already has one macronuclear anlage. This proves that, *after* the discussed cell division in the climax, the one micronucleus forms, by two subsequent divisions, four micronuclei, two of which become macronuclear anlagen which are distributed by the succeeding cell division to each of the two daughter cells.

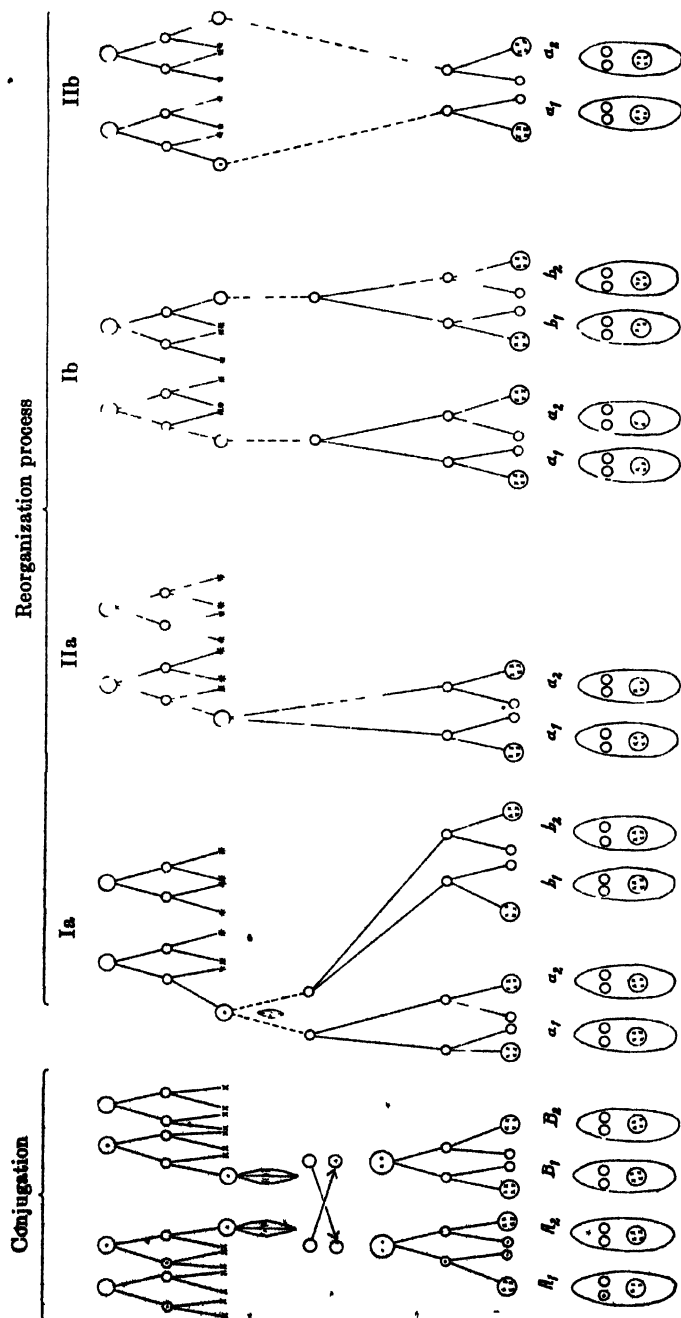
In figure 16a (pl. 2) is given a more advanced stage of one of the cells immediately after division. This anterior cell (a) has just emerged from the climax, as is evident from the fact that the formation of the macronuclear anlagen is completed. The sister cell (b) has not undergone the same changes and one micronucleus and several chromatin bodies, of which only five

at the anterior end are figured, can be detected. Figure 39 (pl. 4) shows essentially the same stages, the only difference being that the anterior cell has effected one more micronuclear division before the first reconstruction cell division than 16a.

While the morphological changes which occur after the cell division at the climax are clear, those which take place before this division (i.e., from the formation of eight 'reduction' micronuclei to the distribution of two to sister cells) must be discussed further. In the instances where two micronuclei remain, it is possible that a micronuclear division has not taken place, but that merely a shifting of one of the micronuclei into each of the two newly formed sister cells has occurred. This method of distribution, which gives an equally satisfactory explanation of the observed data, we call Case Ib (text fig. 3). The results of cases Ia and Ib are the same—two cells with one micronucleus and many chromatin bodies at the climax.

Only twice during these experiments has a paramaecium been observed which had a macronucleus half devoid of chromatin, two well formed micronuclei, two macronuclear anlagen and several chromatin bodies. In these cases the latter were so small and homogeneous that they readily could be mistaken for 'reduction' micronuclei. Here it is evident that no division during the climax took place and that the formation of the anlagen has occurred precociously. There are thus two possibilities as to the *origin* of this condition: (a) *One* so-called reduction micronucleus may remain and divide. The two micronuclei thus arising form the anlagen (text fig. 3, IIa). (b) *Two* so-called reduction micronuclei may remain and divide, thus forming immediately the macronuclear anlagen (text fig. 3, IIb). The result in either case is the same—one cell with two micronuclei and two macronuclear anlagen.

Data will later be presented (cf. p. 495) which afford strong *physiological* evidence that a third micronuclear division producing gametic micronuclei (stationary and migratory) must be absent. It is important to note that if no micronuclear division takes place between the last so-called reduction division and the divisions which form the new micronuclei and give rise to the



Text fig. 3 Methods of micronuclear and cell division at the climax of the reorganization process.

macronuclear anlagen (Ib, IIa, IIb), a primary distinction between the morphological phenomena in conjugation and in the process here described is established; i.e., the important micronuclear division which forms the migratory micronucleus and the stationary micronucleus is absent. But we wish to emphasize the fact that if a third micronuclear division occurs, it can equally well be a precocious division in the ascending or reconstruction phase of the reorganization process.

The diagram presented herewith (text fig. 3) summarizes the different cases which are possible from the observed facts. *All the described cases are distinguished from conjugation and autogamy by the absence of the formation of a syncaryon.* In the process only in case Ia already described is it possible that a third micronuclear division at the climax takes place. The difference between conjugation and the process in case Ia consists in the occurrence of one more *cell* division in the latter than in conjugation. The possible variations in cases Ib, IIa and IIb agree in the absence of the third micronuclear division under discussion. Cases IIa and IIb have no cell division before the formation of the macronuclear anlagen, one or two so-called reduction micronuclei remaining in the same old cell. Case IIb is peculiar in the shifting of one micronucleus into each new cell.

But from a study of all the observed sequences of generations at this period there is some evidence that case Ib presents the usual method of nuclear changes at this period. Line VI_d had four and Line VI_b had three animals in the reorganization process, all of which were at the first stage of the ascending phase—formation of the macronuclear anlagen. This one fact is crucial evidence that a *cell division* has occurred before the formation of the macronuclear anlagen, but no direct *observation* can be presented which determines whether the discussed micronuclear division has or has not taken place, because one spindle in a cell filled with chromatin bodies without a macronucleus or with a macronuclear membrane devoid of chromatin can represent either: (a) the discussed third micronuclear division or (b) the division which forms in cases Ia, Ib, and IIa the two new micronuclei which in the next division form the macronuclear anlagen.

Figure 40 (pl. 4) shows an actual cell to illustrate these statements. The animal has many chromatin bodies and two micronuclei which have just divided. Only because the *fate* of the cell (VIh, 4355th generation) is known, is it certain that these two micronuclei are the two micronuclei which, by another micronuclear division, form four micronuclei, two of which become macronuclear anlagen. If the cytology of the cells related to this cell were not known, its two micronuclei could be interpreted as the products of the discussed third division, which, if it occurs, should take place *before* the cell division in the climax. *We have in our pedigreed lines no indication of its occurrence.*

Only on the assumption that this important division in the climax has different morphological structures from the other divisions of the reorganization process would it be possible to recognize it. However since we have only seen small elongated spindles during the *ascending* phase there is no reliable morphological criterion to determine the presence of the third micronuclear division. One might expect that the condition of the chromatin bodies would give some indication of the extent to which the process has advanced and therefore of the number of micronuclear divisions which has occurred. But this is not a positive criterion because the disintegration of the chromatin bodies does not progress with equal rapidity in all cells.

We are certain, from the *combined* evidence from our cytological study and from physiological data which will be presented later, that the micronuclear division which would be comparable to that which forms the stationary and migratory micronuclei in conjugating animals is absent in the reorganization process. In *conjugation* the reorganization of the nuclear apparatus is consummated by the syncaryon. In the *reorganization process* the so-called reduction micronuclei or their descendants give rise to the new nuclear apparatus. This significant feature is obviously of great importance from the standpoint of the theoretical interpretation of the reorganization process which we describe.

A brief survey of all the stages from the climax which we have figured in the plates substantiate, we believe, the cytological

data which we present and our interpretations of them. The third micronuclear division is absent; two 'reduction' micronuclei remain which are shifted into two cells. Figures 15, 16 and 39 show the cell division in the climax; figure 41 gives a single cell which has completed that division; figure 40 shows the next micronuclear division preceding the formation of the macronuclear anlagen; figure 16, anterior cell, figures 17 through 24, and figure 39, anterior cell, give the formation of the two macronuclear anlagen and the micronuclear changes in each stage. The distribution of the macronuclear anlagen is shown in figures 25, 42, and 43.

C. ASCENDING PHASE

The ascending phase of the reorganization process, which is the longest of the three, extends from the formation of the macronuclear anlagen to the restoration of the typical paramaecium cell with one macronucleus and two micronuclei. It is identical with the periods F and G which Maupas describes as the first divisions after typical conjugation in *Paramaecium aurelia* ('89, p. 221).

Two micronuclear divisions occur in a very short time, half the products of the second forming the two macronuclear anlagen. The two untransformed micronuclei divide again and the first cell division ensues. The following divisions of the cell are exactly similar to typical vegetative divisions and can only be distinguished by the fact that the involution of the chromatin bodies is in progress.

The details of this period are remarkable in various ways. Figures 17, 18, and 19 (pl. 2) give details of the formation of the macronuclear anlagen. The chromatin bodies are omitted from the drawings. These three preparations are counter-stained with eosin, which, according to Calkins ('07, p. 383), stains the non-chromatic parts of the micronuclei of *Paramaecium caudatum*. The posterior micronucleus (fig. 17) has just divided and of the products of this division one remains a typical micronucleus while the other shows the beginning of the development of a micronucleus into the fundament of the macronucleus.

Chromatin has just separated from the achromatic threads but still retains its micronuclear structure. The next step in forming the anlagen is the distribution of the threads and the arranging of the coarser chromatic granules underneath the membrane. This step has been effected in the anterior anlage shown in the same figure. Figure 18 gives a clear idea of the completed anlagen and the two micronuclei from which they arose. Here the granules have become smaller and the plasmatic character of the structure is more evident because its staining capacity for eosin has increased. The micronuclei show the chromatin surrounded by a plasmatic layer highly stained with eosin. In figure 19, the next division, which is the first in the new rhythmical period of the life history, is completed in one pair of micronuclei and is in progress in the third micronucleus which is lying just above the one chromatin body shown in the figure, though many were present in the cell. All the chromatin bodies are represented in an animal from Line VI (fig. 21, pl. 2). Nearly all are more or less spherical and the dissolution of the chromatin is evident. Two macronuclear anlagen, lying close together, and stained a reddish-blue, show marked paucity of chromatin. The two functioning micronuclei are lying at the opposite ends of the cell, while a spindle, in which chromatin has passed to the two poles, is also visible. This spindle represents the same formation as that figured by Maupas in a conjugating animal ('89, pl. 13, fig. 27). Above one of the chromatin bodies is seen a small chromatin granule which may be interpreted as a degenerating micronucleus.

The disintegration of the chromatin bodies, which are now often surrounded by a clear area, is also progressing in the animal represented in figure 20 (pl. 2). The macronuclear anlagen have lost their round contour and appear more or less irregular while the two micronuclei are ready for division. The micronuclear divisions are far more advanced in the specimen shown in figure 22 (pl. 2) and the two spindles present indicate an ensuing cell division. One micronucleus is apparently degenerating. Large vacuoles are usually present in the cytoplasm during this period.

The last period of the ascending phase is characterized by the disappearance of all chromatin bodies. Figure 25 (pl. 3), Line VIIb, represents an animal, after the first cell division following the origin of the macronuclear anlagen, showing two micronuclei at the left, twenty chromatin bodies and several large vacuoles. A single new macronucleus is visible. The other macronuclear anlage, of the two in the parental cell, has been distributed to the sister cell which was kept to continue Line VIIb. This method of distribution of the two macronuclear anlagen to each of the new animals is the same as in typical conjugation of *Paramecium aurelia*. Maupas ('89, p. 222, pl. 13, figs. 23-27) gives a full account of this distribution. Hertwig ('89, p. 38) shows that one of the products of each of the two dividing micronuclei becomes a macronuclear anlage and the other a micronucleus, but this is not evident from his figure (pl. 3, figs. 9 and 10). The destruction of the old macronucleus occurs in most Infusoria during and after conjugation by the formation of ribbon-like structures which are finally resolved into chromatin bodies and disappear. This is the method which obtains in *Paramecium caudatum* and *Paramecium aurelia* (Maupas, Hertwig) and in *Paramecium putrinum* (Doflein). But the accounts of this stage given by Maupas, Hertwig, and Calkins do not afford details of the ultimate fate of the chromatin bodies, since they merely mention that, by the formation of the daughter cells, these bodies decrease in number and their remnants become pale and disappear in the cytoplasm. Collin ('12, p. 223) however, gives a thorough account of the old macronucleus in *Acineta papillifera* and figures (text-fig. 63) the gradual resorption of the chromatin remnants in the cytoplasm. A different fate of the macronucleus was observed by Ubisch ('13, p. 72) in a study of *Lagenophrys*, who states that the chromatin bodies, which appear very similar to micronuclei, are actually ejected from the macrogamete after the formation of the syncaryon. The extruded chromatin bodies were finally observed between the animal and its test.

The total disappearance of the chromatin bodies in the reorganization process of *Paramecium aurelia* is thoroughly described on page 455. It is distinguished from the similar stage in con-

jugation by the fact that chromatin ribbons are not formed. There is no evidence that the chromatin bodies are ejected from the cell or that the remnants fuse with the new macronucleus. After the reorganization process the totally dissolved material of the old macronucleus probably remains, at least temporarily, in a changed chemical form in the cell.

From our studies of conjugation in animals derived from the main culture of the pedigreed race of *Paramecium aurelia* we can show clearly that the first division after conjugation distributes to each cell two micronuclei and one macronuclear anlage (fig. 33, pl. 3). There is no degeneration of nuclear material as Maupas ('89, p. 204) and Klitzke ('14, p. 8) have described in *Paramecium caudatum*. Maupas as well as Klitzke state that eight micronuclei are formed from the syncaryon by two mitoses quickly following each other, four of the resulting micronuclei forming the macronuclear anlagen, three degenerating and one remaining as the definitive micronucleus of the reorganized cell. By two cell divisions, accompanied by divisions of the micronuclei, the typical vegetative stage in *Paramecium caudatum* is restored. However, Calkins and Cull ('07, p. 387) do not mention a degeneration of nuclear material in this stage but state that four of the eight micronuclei are transformed into macronuclear anlagen and, by two cell divisions without division of the micronuclei, four typical vegetative animals result. Dofflein in his account of *Paramecium putrinum* gives the same interpretation as Maupas and Klitzke, i.e., three micronuclei degenerate and the four potential macronuclear anlagen are distributed by two cell divisions to four animals. Hamburger ('04, p. 223) states that in *Paramecium bursaria* two micronuclei and two macronuclei are formed which are distributed in one of the two following ways to the sister cells which arise: either one micronucleus and one macronuclear anlage pass to each new cell; or one macronuclear anlage and one micronucleus (the product of an additional micronuclear division) pass to one sister cell, while one macronuclear anlage and the other micronucleus (resulting from the extra micronuclear division) passes to the other sister cell. The normal vegetative state of *Paramecium bursaria*,

completely free from disintegration products of the old macronucleus, is often not completed before the third division. All descriptions of the behavior of the paramaecium cell of the different species after the formation of the syncaryon agree in the origin of four or eight new micronuclei, but these micronuclei resulting from the syncaryon are either all used in the further development of the cell (Calkins and Cull, *Paramaecium caudatum*; Maupas, Hertwig, Woodruff and Erdmann, *Paramaecium aurelia*; Hamburger, *Paramaecium bursaria*), or some degenerate (Maupas, Klitzke, *Paramaecium caudatum*; Doflein, *Paramaecium putrinum*). Therefore the weight of evidence at hand indicates that the latter alternative is due to a wrong interpretation. Similarly in the process under discussion in *Paramaecium aurelia* there is no evidence of a wasting of chromatin material in this period and its morphological features are identical with all statements of the nuclear changes in typical conjugation in this species.

The observations of Hamburger on *Paramaecium bursaria* prove that the first micronuclear division after the development of the anlagen may occur either in the old cell or later in the two daughter cells which arise from it. Before the first cell division in the reorganization process two spindles are formed (fig. 22, pl. 2) which indicate that the first cell division after the formation of the macronuclear anlagen restores the normal nuclear apparatus of the cell. During the next two generations the remnants of the old macronucleus are completely eliminated. This is effected by complete dissemination in the cell of the chromatin bodies (fig. 27, pl. 3) in the form of cloud-like masses of faintly staining chromatin granules which soon undergo total involution in the cytoplasm. This disintegration process does not occur simultaneously in all the chromatin bodies as is shown in the figure under discussion in which four bodies are still intact.

The animals which have undergone their first vegetative division are filled with vacuoles as shown in an animal from Line IIIb, 4313th generation (fig. 27, pl. 3). The macronucleus has assumed the peculiar spherical form which it frequently shows immediately after cell division. The climax was in the 4312th

generation and the involution of the chromatin bodies was completed in the 4316th generation. Of the eight cells which were formed by the three following divisions (4313 to 4316), four in the 4316th generation were without chromatin bodies. The preceding figure (fig. 26, pl. 3) represents an animal from Line VI, 4185th generation. Two divisions have occurred since the climax at the 4183d generation. Here the chromatin bodies which still remain have not lost their original shape, but if one considers the four division products which result from the divisions 4183 to 4185 then it is evident that a second method of decreasing the number of chromatin bodies is by their distribution to the daughter cells. As an example of this distribution the following case may be cited (cf. text fig. 8 for sister line):

Line VI	4183d generation	has 19 chromatin bodies
Line VI	4185th generation, animal a	has 6 chromatin bodies
Line VI	4185th generation, animal b	has 5 chromatin bodies
Line VI	4185th generation, animal c	was not observed
Line VI	4185th generation, animal d	was kept alive to continue the line

Two further divisions took place in twenty-four hours:

Line VI	4187th generation, animal a	has still 3 chromatin bodies (fig. 29, pl. 3)
Line VI	4192d generation	has 1 chromatin body

Therefore the ascending phase of the process can be followed in nine generations; that is, from the 4183d generation, which is at the end of the climax, to the 4192d generation which is a typical vegetative paramaecium cell except that one chromatin body still persists. Since the beginning of the process was observed at the 4182d generation, it is evident that the nuclear changes in this case extended over ten generations. The typical reduction of the division rate at the climax of the process to one division in approximately thirty-six hours occurred in this case at the 4182d to 4183d generation, and the increase in the rate to three divisions in twenty-four hours which characterizes the ascending phase of the process occurred at the 4189th to 4192d generation.

When the second or third division after the formation of the macronuclear anlagen has occurred the new macropucleus has

assumed its typical macronuclear characteristics. The elongated form is the prevailing one in the interval between two cell divisions, the broad axis is relatively short, while the few granules within the membrane are small. The appearance of an old and new macronucleus is markedly different and the two stages cannot be confused (compare figs. 3, 4 and 5, pl. 1, with figs. 28, 29 and 30, pl. 3). Figure 29 and figure 30 (pl. 3) show the new macronuclei. The animal portrayed in the former is from the above-mentioned Line VI in the 4187th generation. One large and two small chromatin bodies are visible. The latter shows a cell which was taken from the main culture in the 1201st generation. The staining capacity of the macronucleus is very marked; the micronuclei present no unusual features; and two chromatin bodies still persist. A dividing animal from the main culture at the 1432d to 1433d generations (fig. 28, pl. 3) further illustrates the distribution of the chromatin bodies. This animal has undergone four divisions during the previous twenty-four hours and is now just completing the fifth cell division, while the micronuclei have divided precociously for a sixth cell division. This affords another example of the acceleration of the division rate which is characteristic of the ascending phase of the process.

The ensuing divisions in the rhythmical period efface the last trace of the chromatin bodies and, therefore, of the reorganization process. In conjugation, as has been shown by Maupas and Hertwig, the chromatin bodies undergo the same fate at the same time. The reorganization of the cell is completed.

In the description of the process nearly all the examples presented have been animals from Sub-culture IE (4020th to 4359th generation) bred for this particular purpose under constant environmental conditions. As already described in the section on technic, animals had been preserved at irregular intervals throughout the first six years of the life of the main Culture I and on the basis of these it was early stated ('08) that profound nuclear changes occur which cannot be interpreted as abnormal and which demand further study. The character and significance of these changes obviously could only be determined by such an intensive daily study of all the available animals as the pres-

ent paper involves. It is important, however, at this point to emphasize that the process herein described is not a phenomenon peculiar to the organisms of this culture after the 4020th generation, but that it was in progress throughout the life of the race. For this purpose various specimens from earlier generations are presented in the plates. For example, figure 23 (pl. 2) represents an animal from the main culture on May 1, 1910, at the 1755th generation with its nuclear apparatus in a characteristic stage of the process, i.e., the formation of the macro-nuclear anlagen. This should be compared with figure 20 (pl. 2) which shows an animal which is a descendant of the former after a lapse of 2329 generations, i.e., it is in the 4084th generation. The similarity of nuclear conditions also is evident in figures 21 and 22. Cases such as this could be multiplied but for brevity it will suffice to note that among the preserved material there are specimens in the 426, 910, 1201, 1452, 1498, etc., generations which exhibit reorganization stages in all respects the same as those the animals have shown during the past six months of study. Consequently, when convenient, these have been figured to illustrate the process.

IV. CYTOLOGICAL DETAILS OF SERIES OF PEDIGREED CELLS IN THE REORGANIZATION PROCESS FROM SUBCULTURE IE

In the preceding description of the cytological changes in the process the data from all lines were presented together in order to afford a composite picture of the nuclear phenomena as they appeared from the entire culture. It is the purpose of the present section to resolve this picture into its component parts in order to emphasize the exact sequence of events as actually observed in individual pedigreed series of animals from *single* lines and thus to demonstrate that our description of the process is not derived merely by combining isolated stages found at different times in the different lines, but by combining the data from a large number of *series* of animals whose genetic relation with each other was exactly known—each series showing a considerable part of the process. In combining these series there

was little chance for error for their cytological phenomena frequently overlapped.

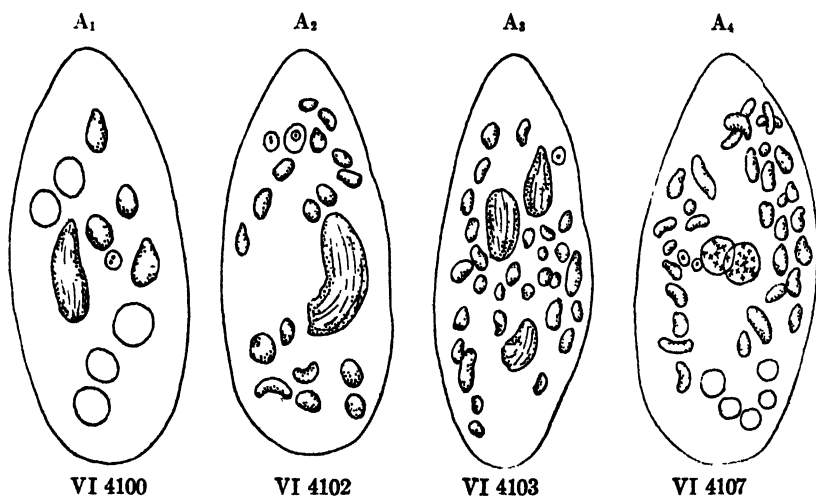
As has already been indicated, it is obviously impossible to study every animal every day and so have a complete picture of the animals of a single line at any one time, since one cell must be maintained if the line is to be kept alive. In certain cases it was deemed best to keep more than one animal alive in order to further safeguard the life of the line because death sometimes occurs during the process. However, it has been possible actually to determine the entire sequence of events by preserving all of the animals of certain lines at selected periods in the process. The fact that the process occurred nearly synchronously in the various lines and at comparatively regular intervals rendered this procedure particularly valuable.

A. SERIES OF CELLS FROM LINE VI AND ITS DERIVATIVES

Line VI at the 4057th generation afforded the first proof of the reconstruction of the cell, two characteristic macronuclear anlagen and twenty-four chromatin bodies being present. The animal is in the beginning of the ascending phase of the process. In the 4060th generation the process was completed as the animal does not show any macronuclear remnants. The large number of typical animals which followed in this line for nearly fifty divisions was finally interrupted by the appearance of a cell at the 4094th generation which has two chromatin bodies. Its sister cell (4094₂) has no signs of the beginning of the process and the following generations up to the 4102d generation showed no further traces of reorganization. This indicates that the cells when the critical period is approaching may start the process but not carry it to completion. The total reconstruction of the nuclear apparatus took place between the 4100th and 4107th generation. Text figure 4 gives two stages of the process; the disintegration of the old macronucleus in A₁ and A₂ and A₃ and the formation of the new macronuclear anlagen in A₄. In the 4100th and the 4102d generation the macronucleus is merely eliminating chromatin bodies while in the 4103d generation the

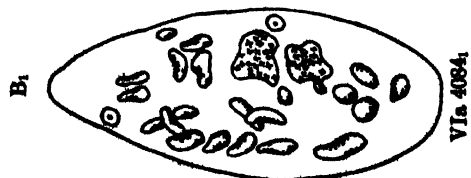
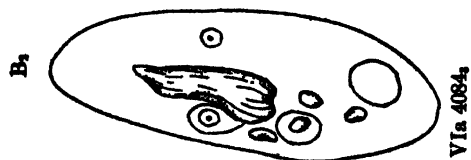
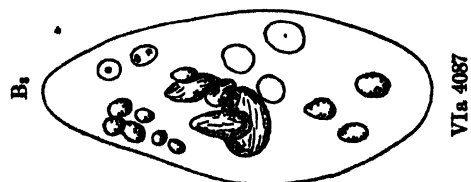
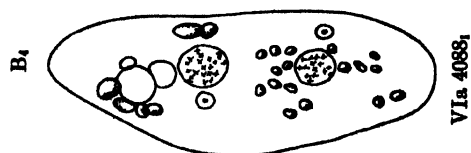
individuality of the macronucleus is destroyed. The animal in the 4105th generation was lost. The cell in the 4107th generation had already formed two macronuclear anlagen and numerous chromatin bodies. One of the latter was still present at the 4110th generation so that in this case the process persisted over about nine generations.

The next critical period began at the 4140th generation. As already mentioned, Line VI made an abortive attempt at the process in the 4094th generation by the extrusion of a single



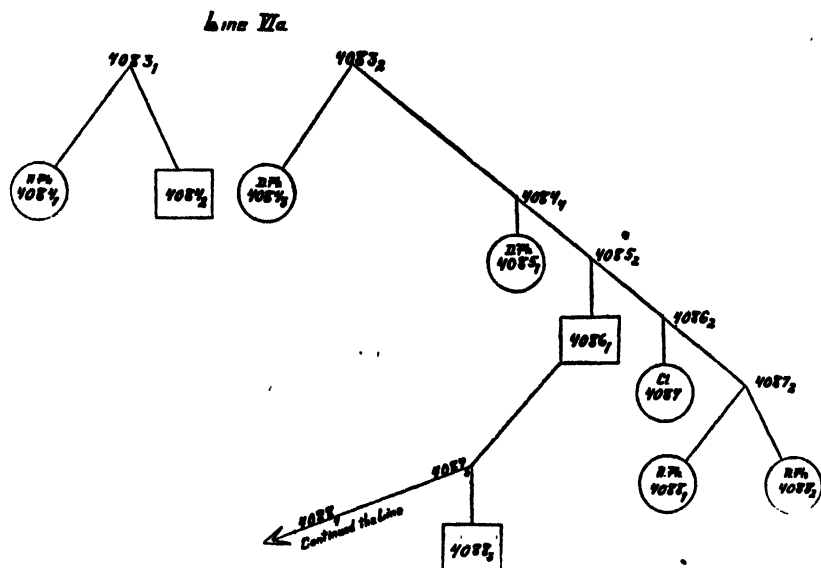
Text fig. 4 Conventional signs, same as in text fig. 1

chromatin body. Line VIa, which was branched from Line VI at the 4068th generation, showed stages of the process from the 4084th to the 4089th generation so that the time of appearance of the process in Line VIa coincides closely with the precocious trial in Line VI at the 4092d generation. In Line VIa the formation of the macronuclear anlagen took place in the 4084th generation (see text fig. 5, B₁). The animal 4084₂ was not preserved but 4084₃ in the descending stage is shown in text figure 5, B₂. Cell 4084₄ continued the line. This cell must have been in the same stage of the reorganization process as 4084₃, because



Text figure 5

in the following generations the process is still going on from the 4084th to 4088th generation (Line VIa, text fig. 5, B₁, B₃, B₄, B₅). Therefore, the process does not progress exactly synchronously in sister or cousin cells. This is clear from the diagram, text figure 6. The stages of the process in Line VIa represented in text figure 5 show the climax and the ascending phase (B₁, B₄, B₅). Cell 4087 is about to form the macronuclear anlagen. Their formation took place in cells 4088₁ and 4088₂ (B₄, B₅).



Text fig. 6 Diagram showing a case of nonsynchronism of the reorganization process in closely related cells.

The fact that the sister cells show slightly different stages in the process of course has no significance from the standpoint of the synchronal appearance of the process in sister lines; that the latter occurs is apparent from tables 1, 2 and 3 (pp. 462-463) which give the occurrence of the process in the lines branches from Line VI. Thus in VIa the process was going on in the 4084th generation, in VIb in the 4092d generation. An examination of table 1 shows that the process was observed in Line

TABLE 1

Showing the occurrence of the reorganization process in Line VI and its derivatives, Culture IE

PERIODS =	4020-4050 GEN.	4050-4100 GEN.	4100-4150 GEN.	4150-4200 GEN.	4200-4250 GEN.	4250-4300 GEN.	4300-4350 GEN.	4350-4400 GEN.	REMARK.
VI		4057	4104	4183	4231				Died in process
4020-4231			4140						
VIa		4064							Died
4068-4095									
VIb		4092		4174					Killed in process
4083-4174									
VIc			4101	4177					Killed in process
4099-4178									
VIId				4180					Killed in process
4178-4180									
VIe				4181					Killed in process
4178-4181									
VIIf				4183	4229				Killed in process
4178-4228									
VIg				4184					Killed in process
4180-4186									
VIh					4236		4315	4355	Killed in process
4189-4358									
VII						4297			Killed in process
4240-4297									
VIj*							4312		Killed in process
4306-4315									
VIk*									Killed during low division rate; animal not in process
4325-4336									
VII*								4355	Killed in process
4348-4355									

* Sub-lines j, k, l were branched from i.

VI in the 4104th generation, in VIc in the 4101st generation. Further evidence of the synchronal occurrence of the process is afforded by the next period in table 1 which shows the process in Line VI at the 4183d generation and in six branches from this line (VIb, VIc, VIId, VIe, VIIf, VIg) between the 4174th and 4184th generation. These facts are substantiated by further data as presented in the graphs of the division rate.

The third repetition of the reorganization process in Line VI took place between the 4140th and 4146th generation. This was relatively early since the second occurrence of the process

TABLE 2

Showing when the reorganization process occurred in Line III and derivatives, Culture IE

PERIODS =	4020-4050 GEN.	4050-4100 GEN.	4100-4150 GEN.	4150-4200 GEN.	4200-4250 GEN.	4250-4300 GEN.	4300-4350 GEN.	4350-4400 GEN.	REMARKS
III 4020-4358		4065		4189	4237		4315		Killed at end of experi- ments
IIIa 4246-4275						4271			D i e d after process
IIIb 4304-4321							4313		Killed; animals small
IIIc 4327-4346									Killed; division rate low, not in process

was at the 4104th generation. The ascending phase of the process was completely secured (text fig. 7). Cell C_1 (4140th generation) has two macronuclear anlagen; C_2 (4141st generation), resulting from one division, has one macronuclear anlage; C_3 (4142d generation) shows the distribution of the chromatin bodies in the daughter cells.

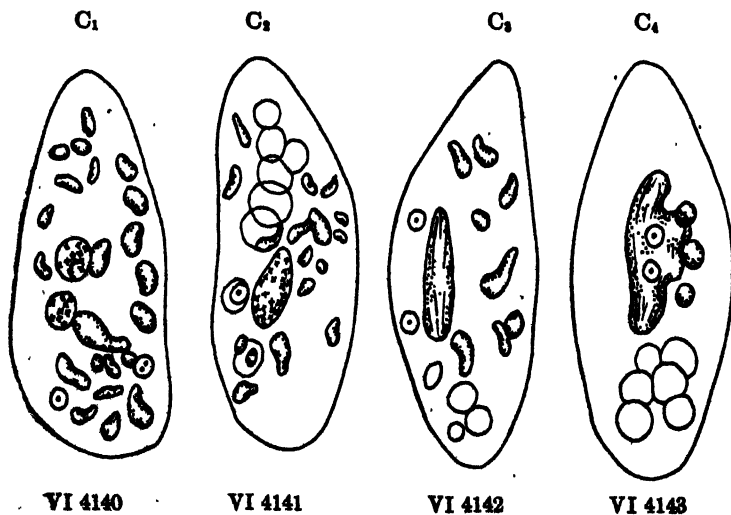
The next occurrence of the process in Line VI, represented in text figure 8, gives nearly a complete picture of each stage in the process in a *single* line. The animal (4180-4181) just dividing was typical, while the following cell (4181-4182) shows simply the extrusion of one chromatin body and the remnant of a

TABLE 3

Showing when the reorganization process occurred in Lines I, II, IV, and V, Culture IE

PERIODS =	4020-4050 GEN.	4050-4100 GEN.	4100-4150 GEN.	4150-4200 GEN.	REMARKS
Line I from 4020 to 4057 gen.	4038				Died in process; last animals small due to abnormal division; lived 4 days without division.
Line II from 4020 to 4172.....	4021	4069		4172	Line killed in process
Line IV from 4020 to 4075.....		4066			Animals small; died
Line V from 4020 to 4170.....	4023		4115		Killed; not in process

division spindle. This proves that in generation 4182 the complete extrusion of chromatin bodies will be completed and the 'reduction' divisions of the micronuclei and their degeneration must also occur here during the thirty-six hours that this cell persists as an individual. The low division rate at this point indicates that the profound nuclear changes temporarily inhibit the reproductive activity. The animal undergoing the next division (4182-4183) was not preserved but evidently would have shown each of the arising cells in the climax of the process



Text figure 7

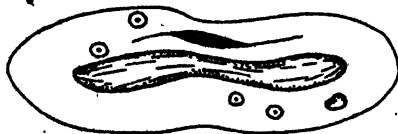
with one or two micronuclei and numerous chromatin bodies. However, one of the completed products of this division, 4183d generation (D₁), shows the two macronuclear anlagen completely formed. The next stages (4185₁, 4185₂, 4187) show the distribution of the chromatin bodies. Cell 4184 was not preserved but evidently it had one macronuclear anlage, so from generation 4182 to 4192 (D₇) with one chromatin body, we have the process characterized physiologically by the lowering of the division rate in the 4182d to 4183d generations when reproduction was deferred for about thirty-six hours, and then the sudden accel-

D₁



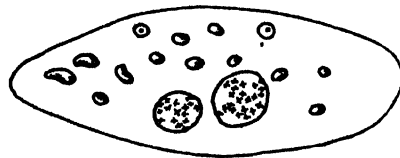
VI 4180-4181

D₁



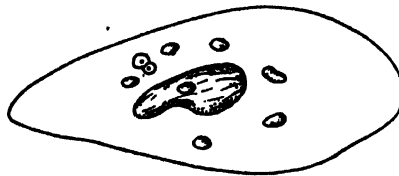
VI 4181-4182

D₁



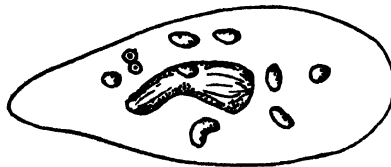
VI 4183

D₁



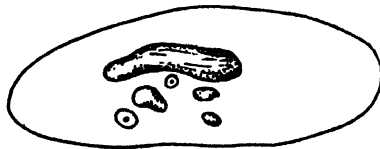
VI 4185,
Text figure 8

D₁



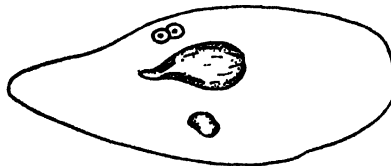
VI 4185,

D₁



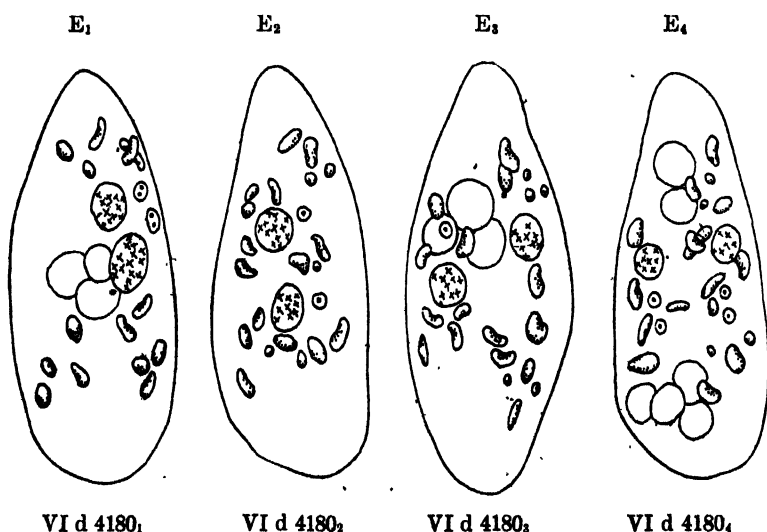
VI 4187

D₁



VI 4192

ation of the fate at the 4187th to 4192d generation when three divisions occurred in the twenty-four hours. The morphological characteristics for the single periods are well established as text figure 8 (D_1 to D_7) proves. The descending phase of the process is of the shortest duration and this makes it particularly difficult to secure all the stages—several coming and going in a single generation. On the other hand the ascending phase is the longest and, therefore, the stage most often seen in all lines is that with two macronuclear anlagen. The synchronism of the process in Lines VI, Vīb, Vīc, Vīd, Vīe, Vīf and Vīg made it possible

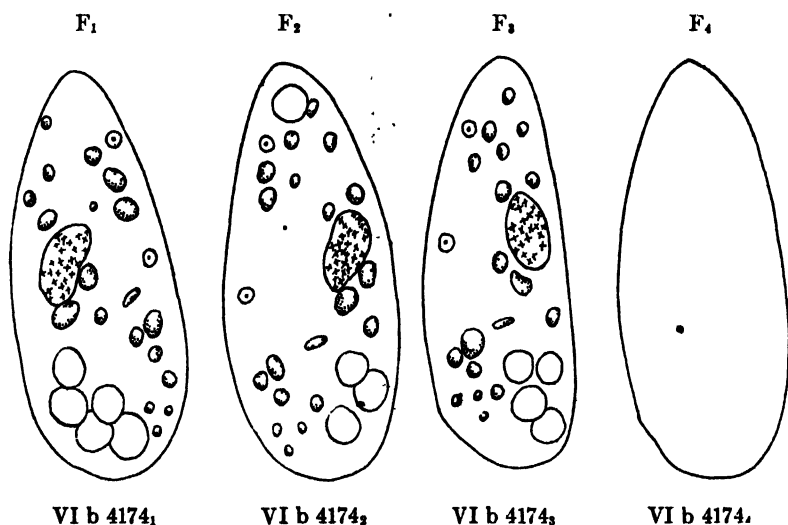


Text figure 9

to kill whole lines undergoing the process and in this way some of the most interesting stages were secured.

Text figure 9 (E_1 to E_4) gives four cells (line Vīd) all at the same stage with the formation of the two macronuclear anlagen completed. The next stage will be the distribution of the two macronuclear anlagen into two sister cells. This stage is simultaneously completed in four cells of Line Vīb at the 4174th generation. Text figure 10 (F_1 to F_4) shows three of these cells

which result from two divisions in forty-eight hours. The fourth animal (F_4) was lost. Since each of the three animals preserved has one macronuclear anlage it is certain that each of the two parent cells in the 4173d generation had two macronuclear anlagen, one of which passed to each daughter cell at division. The fourth animal (F_4) which was lost during preparation obviously must have been in the same stage as those figured. The next text figure, 11 (G_1 to G_3), shows again that at least seven or eight generations are necessary to complete the process. The



Text figure 10

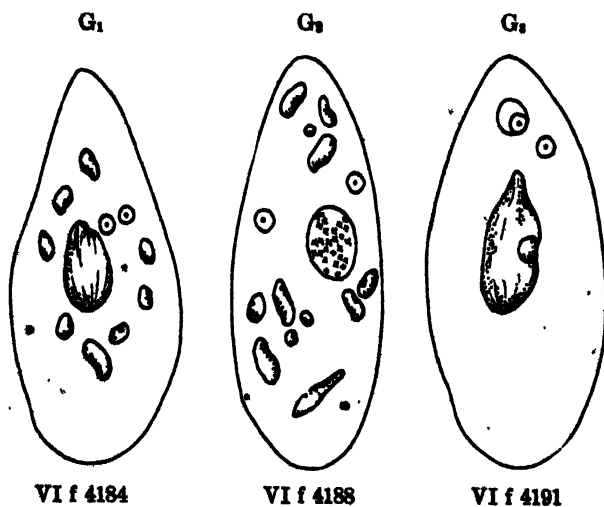
animals are from Line VI f in the 4184th, 4188th, and 4191st generation and show the extrusion of the chromatin bodies from the old macronucleus, the new macronuclear anlagen and the nearly reorganized paramaecium cell.

The last occurrence of the process in Line VI extended from the 4229th generation to the death of the line at the 4231st generation from the accidental infection of the culture medium with a deleterious strain of bacteria, as mentioned on pages 478 and 489. Five times in 211 generations the cell reorganized its nuclear

apparatus by the process; six times the division rate (see p. 478) was relatively low. Five times this was synchronous with the process; once with bacterial infection.

The process occurred in Line VI and its sublines at intervals of about forty to fifty generations. For example, Line VI had the process in progress at the 4231st generation, Line VI f at the 4229th generation, and Line VI h at the 4215th generation.

The stages of the process which occurred in Line VI i in the 4279th generation and Line VI h in the 4315th and 4355th gen-



Text figure 11

eration, and in Line VI j in the 4312th generation did not show any new features. Simultaneously with Line VI h, Line VII underwent the process in the 4355th generation. Line VI k was killed after eleven generations, when the division rate fell slightly, with the expectation of securing early stages of the process. However the process was not found but judging by closely related lines it would have appeared about twenty generations later. Line VI together with its twelve branches and subbranches exhibited the process a total of twenty-one times from the 4020th to the 4355th generation at clearly recognisable periods, showing that

there is an inherent tendency for sister lines to undergo the process synchronously. The significance of this will be considered later.

The death of Line VI during the process indicates a fact which we have noted several times in these experiments and one to be expected when the complexity of the reorganization process is appreciated. The animals at this time are in a relatively susceptible condition and consequently more readily succumb to slight environmental changes. It is undoubtedly at such periods that many cultures not bred under the most favorable conditions have become exterminated, and the attending cytological conditions have naturally been interpreted as the results of degeneration.

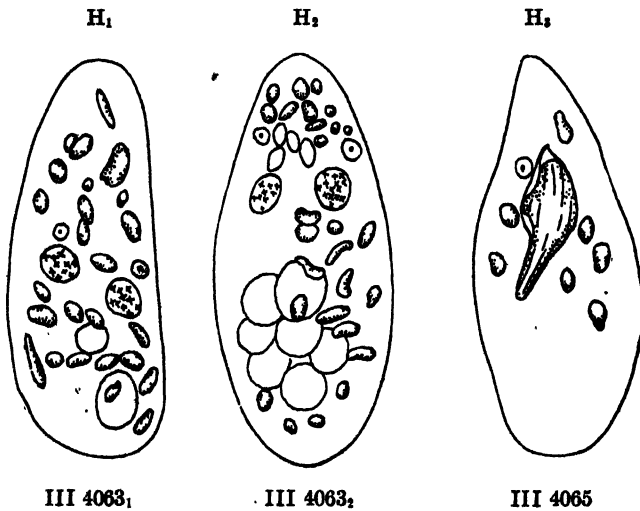
The cells during the process seem to be more opaque than usual, their breadth is relatively greater, their total volume is somewhat increased and their movements are considerably more sluggish. Cells with this appearance defer division for about thirty-six hours and are at the climax of the process. The animals at this time are more difficult to handle with a pipet owing to the fact that they have a tendency to adhere to the glass or to minute particles of debris, thus indicating the so-called miscible state noted by Calkins, Erdmann, Popoff and others in conjugating forms. Also, animals in this condition will sometimes burst on transference to fresh culture fluid medium or to a fixing fluid.

Thus, by the continued daily study of the animals and their rate of division one finally becomes able to tell, with a considerable degree of accuracy, the occurrence of the process merely by the use of a low power of a Zeiss binocular microscope.

B. SERIES OF CELLS FROM LINES I, II, III, IV, V

Line III, which was not under such close observation as line VI, had the process at the 4065th, 4189th, 4237th, 4315th generations. Text figure 17 gives the graph of the division rate averaged for five-day periods. One lowering of the division rate occurred at generations 4140 to 4144. During this time the

cells from Line III were not preserved but judging by the division rate of the line and the occurrence of the process at this period in Line VI, it is almost positive that the process took place in Line III at this time. Cell 4148 was preserved and found to be a typical cell without traces of the process. Text figure 12 (H_1 to H_3) give examples of the ascending phase of the process from Line III. The cells 4063₁ and 4063₂ had formed the macronuclear anlagen. Two divisions later, cell 4065 (H_3) had only seven chromatin bodies, showing a reduction of these bodies by



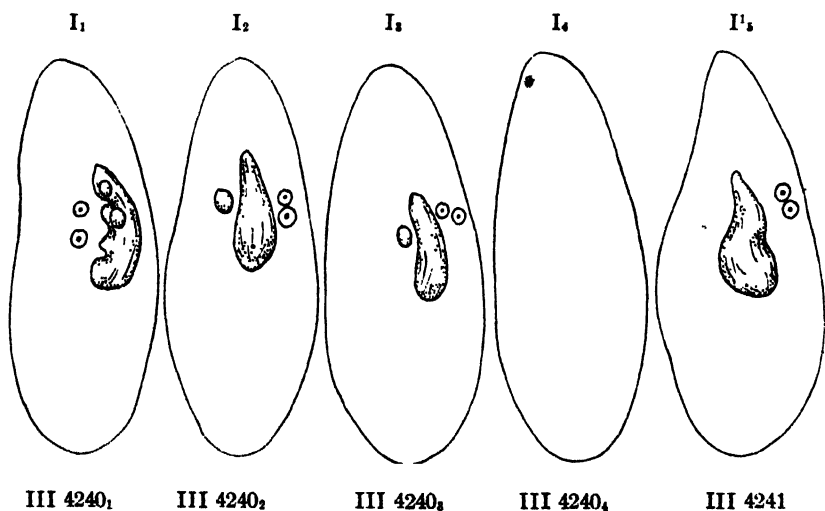
Text figure 12

distribution and absorption from twenty-seven (H_2) to seven during these two cell generations. Text figure 13 (I_1 to I_5) give some of the later stages of the ascending phase. The three animals which could be observed had a few chromatin bodies. Cell 4240₁ had two, 4240₂ one, 4240₃ one chromatin body. Cell 4240₄, which continued the line, gave rise to typical animals at the following division, 4241st generation (I_5).

Text figure 14 (J_1 to J_5) shows the process after the formation of the macronuclear anlagen in cell 4189. After two divisions the new macronucleus is seen in the sister cells 4191₁ and 4191₂.

Thus the distribution of the two macronuclear anlagen took place in the 4190th generation, which affords further proof that *the two macronuclear anlagen are distributed by one cell division to each of the resulting sister cells.*

In Line III and its branches, IIIa, IIIb, IIIc, the process was actually observed six times, while undoubtedly it also occurred several times unobserved, judging by the division rate and the periodic occurrence of the process in sister lines (see table 2 and text fig. 17).

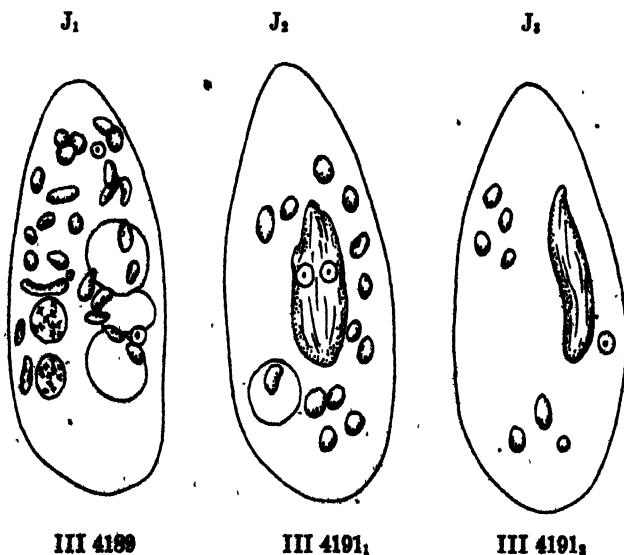


Text figure 13

The Lines I, II, IV, V, which were not branched, show the process a total of seven times, each time at due periods coinciding with Line VI. Some of the stages observed in these lines are presented in the plates as they supplied some of the details of the process as, for example, Line II, 4069th generation (fig. 9, pl. 1) which give a particularly clear idea of the extrusion of the chromatin bodies.

One point which is shown by a study of tables 1, 2, and 3, which give the life history of the twenty-one lines, is the occurrence of death in certain instances during the process. Of these

lines seventeen were killed intentionally, while of the remaining four lines, three died during or shortly after the process. Some of these animals showed irregular divisions resulting in very small cells which apparently lacked the power of growth. It is important here to emphasize that fatalities may occur as a result of faulty reorganization during the process just as has so often been observed to occur in conjugating animals. Both conjugation and the reorganization process, however, are normal phenomena which have no pathological features.



Text figure 14

The detailed discussion of Lines I to VI and their branches and subbranches gives clear evidence that the process, resulting in a reorganization of the nuclear apparatus which simulates typical conjugation, is going on with strictly determined morphological and physiological features at relatively definite periodic intervals of time in the pedigreed race of *Paramaccium aurelia* which is the basis of this investigation.

V. THE REORGANIZATION PROCESS IN THIS RACE AFTER
CONJUGATION AND IN OTHER RACES AND
SPECIES OF PARAMAECIUM

So far it is clear that the nuclear reorganization occurred throughout the life of this race bred under conditions which absolutely precluded the occurrence of conjugation, and the question arises here: Would the process again occur periodically after conjugation had taken place? Animals were accordingly isolated from this race at the 4102d generation and with them a large mass culture was started in a stender dish. Within a few days a number of conjugating pairs were observed and about twenty were isolated. Some of the animals were preserved for details of conjugation in this race, while the descendants of other ex-conjugants were bred on depression slides by the same isolation culture methods as those used in the main culture from which they were derived at the 4102d generation. A study of the animals preserved from these ex-conjugant lines demonstrates that *the same reorganization process was resumed in all the lines within a relatively short time after conjugation.*

More positive evidence could hardly be presented to prove that the process is a fundamental normal periodic phenomenon in the life of this race of *Paramaecium aurelia*.

This being established, the question arises: Is this a peculiarity of this race or does the process occur generally in *Paramaecium aurelia*? This we can also answer conclusively. Erdmann on August 11, 1912, isolated a specimen of *Paramaecium aurelia* from a canal of the river Spree in Berlin and bred its descendants by the daily isolation method on a culture medium of beef extract. Specimens from Erdmann's race, bred by this method which absolutely prevents conjugation, were preserved from time to time and figure 24 (pl. 2) shows one of these in a characteristic stage of the process under discussion, i.e., the formation of the macronuclear anlagen. Thus it is evident that the same nuclear reorganization which has occurred throughout the life of Woodruff's race started with a specimen of *Paramaecium aurelia* isolated at random in America in 1907 also occurred in Erdmann's race similarly isolated in Germany in 1912.

Therefore the data justify the conclusion that this reorganization process is a normal phenomenon and probably *occurs in all races of the species Paramaecium aurelia*.

Most suggestive observations on nuclear changes in *Paramaecium aurelia* were made incidentally by Hertwig during his study of conjugation in this species. This author noted ('89, pp. 74-75) in a mass culture, in which conjugation had not been observed for a long period, certain animals whose nuclear structure apparently indicates isolated steps in the phenomenon which are described in detail in the present paper, and the significance of which is here elucidated.

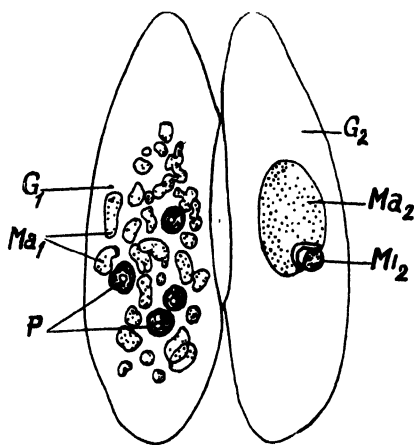
Hertwig grouped the stages which he observed under two general classes, one of which he interpreted as merely a vegetative phenomenon, in which a *new* nuclear apparatus is not formed:

Die zuerst eintretenden Veränderungen besitzen kein Analogon in den Vorgängen einer normalen Entwicklung. Wahrscheinlich zerfällt der Hauptkern erst in grössere dann in kleinere Stücke, ohne das regelmässige Auswachsen in Fortsätze, welches im Lauf der geschlechtlichen Entwicklung der *Paramaecium* eintritt. Ich fand bald 2, bald 4 Nebenkernkerne entweder in Form der ruhenden Kerne oder häufiger in Form von Spindeln, wie ich sie ebenfalls sonst nicht beobachtet habe.

Our data show this to be a mistake. These stages are clearly some steps in the descending phase of the process just before the beginning of the climax.

The second group of changes of the nuclear apparatus described by Hertwig comprised: "Tiere mit vergrösserten Nebenkernen, mit Sichelkernen mit 2, 4 und 8 Spindeln, Tiere, bei denen die Teilung in die Haupt- und Nebenkernanlagen vollzogen war."

From this brief note it is clear that the animals with two and four spindles may possibly represent ordinary vegetative division because the striking disintegration of the macronucleus is not mentioned. We have never discovered an animal with *eight spindles*, but "Tiere, bei denen die Teilung in die Haupt- und Nebenkernanlagen vollzogen war" are apparently identical with those animals which we figure (figs. 21, 22, pl. 2) and represent the important reconstruction of the new nuclear apparatus. If it be true that in Hertwig's culture conjugation had not oc-



Text figure 15

curred (it is impossible to prove this since he employed mass cultures in the work) then it is also true that he discovered at least one of the steps in the process which is outlined in the present paper but obviously he failed to recognize its general fundamental importance. This is evident from the trend of his work and that of his students, on the life history of Infusoria, during the subsequent quarter of a century.

Another question naturally suggests itself at this point: Is the process confined to *Paramecium aurelia*? Figure 34 (pl. 3) is from a pedigreed race of *Paramecium caudatum* which we have studied by the same experimental methods, and shows a characteristic stage in the process. This indicates that it occurs, at least with essentially similar features, in *Paramecium caudatum* also. It will be shown in a later section of this paper that certain of the morphological changes interpreted by Calkins, Popoff and others as degeneration phenomena in *Paramecium caudatum* are in all probability the same process. It may be mentioned here, however, that Doflein ('07) figures an abnormal conjugating pair of *Paramecium caudatum*, one animal of which appears to be a typical vegetative animal while the other shows what he interprets as conjugation nuclear phenomena in a single

animal. From our results we would interpret this as a stage in the ascending phase of the process, because he figures the four macronuclear anlagen typical for *Paramaecium caudatum* and terms them Mikronukleusderivate (see text figure 15, which is a copy of Doflein's figure).

Finally, does the process occur in other genera of Infusoria, and in other classes of Protozoa? Later in this paper we shall discuss the literature relative to this point but it can be stated here that while no positive evidence is extant to answer this question, because pedigreed cultures involving a daily detailed cytological study have not been made, nevertheless, such more or less random facts as may be gleaned from various investigations strongly suggest, at least, that the authors are dealing with comparable nuclear reorganization phenomena.

VI. THE REORGANIZATION PROCESS AND ITS RELATION TO RHYTHMS

Earlier work on this culture has shown that there are rhythms in its rate of reproduction which are not the results of environmental variations, but which are due to some periodic internal phenomena of unknown character.

Minor fluctuations are evident in the division rate of other species of Infusoria bred by the daily isolation method first employed by Calkins. The fluctuations in the culture graphs of Calkins on *Paramaecium caudatum*, Woodruff on *Oxytricha fallax* and *Gastrostyla steinii*, Gregory on *Tillina magna*, and Moody on *Spathidium spathula* appear to be of the same character as those demonstrated in this culture. However, since the authors in the study of the life history of these species have not eliminated the possibility that the fluctuations in the division rate are the results of environmental changes, it is impossible to state positively that the fluctuations observed by them are actually 'rhythms,' though it is highly probable that such is the case.

It is important to emphasize the fact, although it is well known, that relatively slight changes in culture medium or tem-

perature will change the division rate. Indeed it may be said that the rate of reproduction is a function of the environment of the cell—except as the rhythms interfere—and consequently the fluctuations which appear in the graph of the division rate of a culture are not, *ipso facto*, true rhythms, i.e., due to “inherent rhythmical changes in the phenomena of the cell” (Woodruff and Baitsell '11 b, p. 357).

The present cytological study demonstrates the nature of the inherent changes in the cell which have their obvious physiological expression in the rhythms of the rate of reproduction.

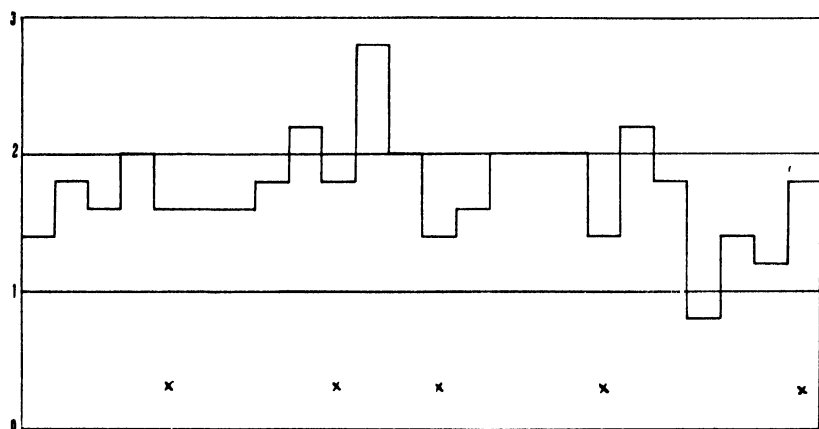
The relations of the process to these fluctuations can be most readily appreciated by a consideration of graphs of the rate of divisions of Lines VI and III of Sub-culture IE, which was subjected to the constant culture medium of beef extract and to practically constant temperature conditions (about 26°C.) within the optimum zone for this race.

The five-day period was adopted in the presentation of our results because this was the method of constructing the graph emphasized in the original study of rhythms in this culture. It is realized, of course, that a five-day period is largely an arbitrary one and that the ideal graph would present the momentary changes in the metabolism of the cell. Data for such a curve being absolutely impossible to secure, it might seem at first glance that the daily record of division would approach most nearly to this ideal condition. As a matter of fact, the twenty-four-hour period is as arbitrary as the five-day period when it is considered that this is a long period when compared with the metabolic changes in the cell and that the daily record, made at approximately 11 A.M., would merely give the divisions actually *completed* during the previous twenty-four hours. For example, let us assume that, at the time of isolation, two animals are present, representing one division during the previous twenty-four hours. The record for that day is one division. One animal is then isolated and it divides within an hour and each of the resulting cells again divide twice before the next isolation. The record for this second day is three divisions, thus the record for the two days shows a different division rate for each day, i.e.,

one division against three divisions, whereas a more true, but not a perfect, picture of the state of affairs is given by the statement that four divisions occurred in forty-eight hours. One might follow this argument to its logical conclusion and assume that the best method of presentation would be to average for considerable periods, e.g., 10 or 30 days, but this obviously would tend to obliterate any fluctuations in the rate which are not of relatively long duration. The adoption of the five-day period was made in recognition of both of these contingencies, and it was of a duration particularly well suited to show the effect of the process on the reproductive rate, because the process extends over about nine cell divisions or a period of about six days. Consequently the effect of the process makes itself evident in the five-day plot. Certain apparent irregularities in the coincidence of the phenomena are, from an actual study of all the data at hand, clearly due to the fact that the five-day period is not ideal.

In a consideration of the relation of rhythms to the reorganization process it will be convenient to consider first the data from Line VI, Subculture IE, since animals from this line were preserved every day of its existence and, during the process, every animal was preserved except the one needed to keep the line alive.

Inspection of the graph of this line shows at a glance five fluctuations in the reproduction rate which would naturally be interpreted as rhythms (cf. text fig. 16) while a study of the cytological condition of the specimens shows that the low point of each of the first four fluctuations is coincident with the profound nuclear changes of the reorganization process. The fifth fluctuation, during which the division rate fell to nearly three-quarters of a division per day, was brought about by the contamination of the medium at the time of transference with a deleterious strain of bacteria, from the effects of which the animals of this line never completely recuperated and succumbed upon the recurrence of the process for the fifth time. Consequently it is evident that four out of the five fluctuations are

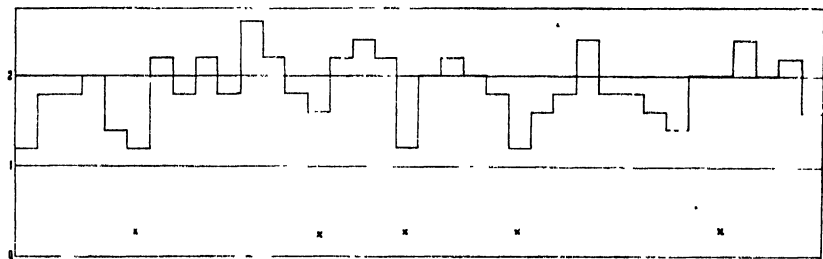


Text fig. 16 Graph of the rate of division of line VI, subculture IF, averaged for five-day periods. The periods during which the reorganization process occurred are indicated by an X.

actually rhythms, i.e., not obviously due to changes in the environment, and at the low point of each of these rhythms the process was in progress. Table 1 shows that the process in Line VI was in progress at the 4057th, 4104th, 4140th, 4183d, and 4231st generation when the line died. These generations represent approximately the climax of the process. Thus it is clear that in the line under consideration the process occurred at intervals of about 40 to 50 generations or about every twenty-five to thirty days, and that the low point between each of the first four fluctuations was coincident with the nuclear reorganization. This evidence from Line VI shows clearly a relationship between the rhythms and the nuclear reorganization.

Cumulative evidence which establishes a casual relationship between rhythms and the process is afforded by a study of Line III which was carried continuously for eight months. Animals from Line III were not preserved every day at the time of isolation (as was the case with Line VI) owing to the great amount of labor involved, but at intervals of several days duration. That is, no attempt was made to have a complete pedigreed series of stages showing its daily cytological condition but merely a broad

view of its nuclear state. By this method the process was discovered at the 4065th, 4189th, 4237th, 4315th generation (cf. table 2). Text figure 17 gives the graph of the division rate of this line, plotted the same as that already presented for Line VI, and shows the periods in which the generations involving nuclear reorganization were discovered. Here again in four out of five cases, the process coincides with the low point in division rate, i.e., between two rhythms. The occurrence of the process of the 4315th generation, however, coincides with the early ascending phase of the rhythm and is an exception for which the data afford no evident explanation.



Text fig. 17 Graph of the rate of division of Line III, subculture IIE, averaged for five-day periods. The periods during which the reorganization process occurred are indicated by an X.

The other main lines were carried chiefly for the purpose of affording a sufficient supply of animals in the process and, after the first couple of months, were preserved only when the process was suspected from the appearance of the cells, the rate of division and the length of time and the number of generations since its last occurrence. Consequently it is unnecessary to consider these lines in detail from the standpoint of the rhythms but simply to emphasize that the evidence derived from them is entirely corroborative of that presented from Lines VI and III (cf. table 3). In our description of the cytological changes in the process it has been shown that the phenomenon extends over about nine cell generations, and that at the climax division is deferred for a period of nearly 36 hours. *Therefore, it is*

evident not only that the reorganization process is coincident with the low point between two rhythms, but also that there is a causal relation between the reorganization process and the rhythms.

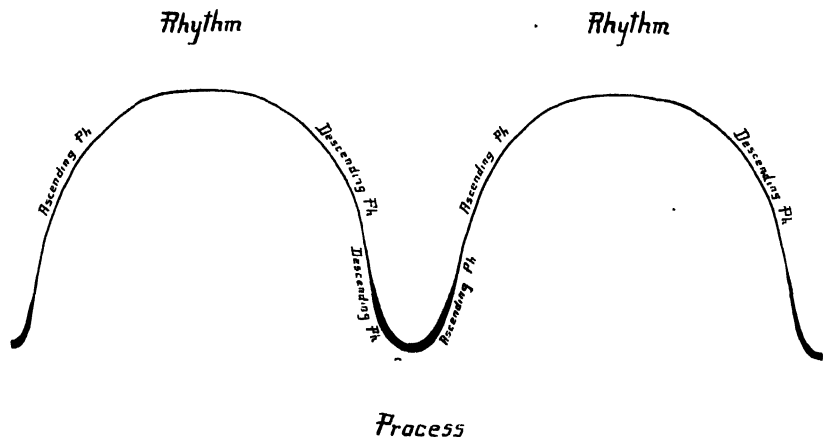
VII THE REORGANIZATION PROCESS AND ITS RELATION TO DEPRESSION PERIODS

The results of the great majority of studies on various species of Infusoria, from the pioneer experiments of Bütschli and Engelmann, have indicated that these forms will not reproduce indefinitely if conjugation between two animals is prevented, but that the race shows signs of lowered vitality accompanied by marked morphological changes which sooner or later end in death. Improved methods of conducting the cultures, however, have enabled succeeding investigators to continue the races longer and longer, until studies on the culture which forms the basis of this paper have shown that under proper environmental conditions *Paramaecium aurelia* can be bred indefinitely without conjugation or artificial stimulation. In other words, there is no evidence of a life cycle, as understood and emphasized by Maupas, Calkins and other authors, which comprises a more or less definite number of cell generations beginning after conjugation with the high potential of vitality of youth and maturity and leading to either old age and death or conjugation and rejuvenescence again.

A critical survey of the literature, however, shows that another type of variation occurs in the life history of Infusoria under culture conditions which must be distinguished from the cycle. In certain cases this has been recognized by the author, but as a rule it has been passed over without comment owing to the fact that only by the daily isolation method of culture is it possible to demonstrate its reality. Apparently Hertwig ('00-'04) from his study by mass culture methods of the life history of *Actinosphaerium eichhornii*, *Dileptus gigas* and *Paramaecium caudatum* was the first to contrast minor periods and deep periods of physiological depression, the latter resulting in death. The lesser periods were marked chiefly by a slight lowering of

the general physiological activities of the cells, and interpreted as the result of relatively slight disturbances of the nuclear condition which were overcome by internal readjustment. In the periods of deep depression, on the other hand, marked signs of degeneration were evident, for which the sole panacea was conjugation. This led Hertwig to the natural suggestion that the nuclear phenomena observed in physiological depression and those which occur at conjugation have fundamentally the same *raison d'être*.

Calkins ('04, p. 424) in his studies on *Paramecium caudatum* noted that the well-marked cycles which resulted in death, unless



Text fig. 18 Diagram illustrating the relation of the reorganization process to rhythms.

drastic methods of artificial stimulation were resorted to, were of about six months duration, while intermediate cycles of less importance occurred at intervals of approximately ninety days, recovery from which took place without purposeful stimulation. In these smaller cycles morphological signs of degeneration were not observed, but in the well-marked cycles cytoplasmic and nuclear changes occurred. These varied somewhat in character at the low points of the various cycles, but in several instances Calkins was able to restore the normal condition by the oppor-

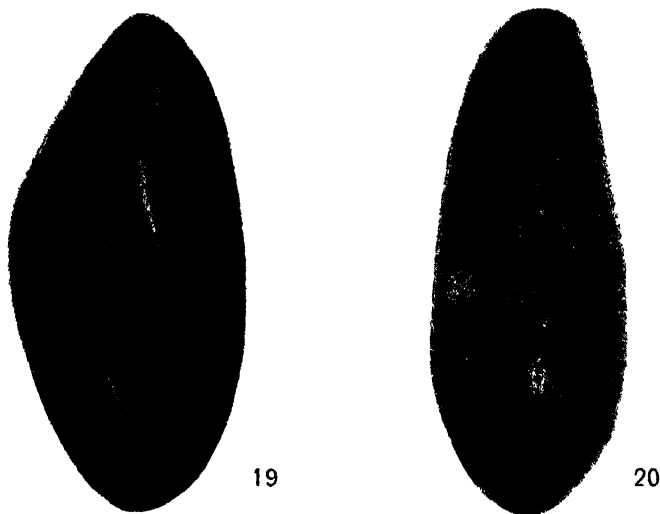
tune use of artificial stimuli. At the last cycle when all the methods of rejuvenation which were tried proved of no avail Calkins observed that the signs of degeneration were apparent in the micronuclei and, therefore, concluded that at last 'germinal death' occurred.

Some very suggestive nuclear conditions are figured by Calkins ('04) from animals in his depression periods. Calkins' figure 15, plate 2, shows an animal of his B series just before its death in the 502d generation, which had been treated with beef extract. He states "that the micronucleus has divided three or more times and the daughter nuclei have accumulated at one end." His photograph does not convince us that these are really micronuclei. If they are they clearly represent an early "reduction" phase of the process as described by us for *Paramecium aurelia*. We are inclined, however, to interpret these 'micronuclei' as chromatin bodies since we have found that in *Paramecium caudatum*, as in *Paramecium aurelia*, this is the method of dissolution of the macronucleus during the process. This figure of Calkins should be compared with our figure 34 (pl. 3) which shows the chromatin bodies in *Paramecium caudatum*. Text figure 19 (Calkins' fig. 16, pl. 2) shows an animal from his

A series in the 602nd generation treated for twenty-five minutes with phosphoric acid. It was transferred to hay infusion and killed twenty-four hours afterwards. The macronucleus is broken into fragments; the micronucleus has divided and one part (left center) seems to be forming a new macronucleus. (This individual offers the only evidence obtained of nuclear fragmentation and reconstruction through artificial means.)

This suggestive comment by Calkins, given merely in his description of plates, apparently hits the mark. We must interpret this depression animal of Calkins, which he attempted to 'rejuvenate' by phosphoric acid, as at the end of the climax of our process with many chromatin bodies and two macronuclear anlagen. The micronuclear condition is not clear from the photograph (compare text figs. 19 and 20, p. 484).

Maupas' observation that in certain hypotrichous forms the micronuclei in some periods may be increased to a number



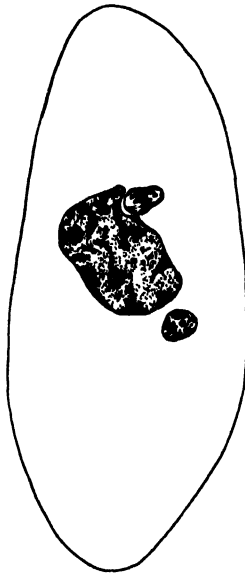
Text figures 19 and 20

beyond that typical for the species was substantiated by Woodruff ('05) who observed marked macronuclear fragmentation as well as a tendency to micronuclear reduplication at periods of low division rate in a culture of *Oxytricha fallax*. The fact that Woodruff was able to 'rejuvenate' the race when apparently on the verge of extinction led him to state that "we are hardly justified in assuming that Protozoa, when dividing at a low rate, with nuclei fragmented, etc., are exactly 'abnormal,'" and "suggests that we are justified in regarding these changes as phases in the life history of Infusoria which occur under certain conditions after a considerable period of vegetative reproduction." It now seems probable that this tentative suggestion points in the right direction. The discovery of atypical conditions, chiefly during the period of deepest depression and throughout the month that it persisted before 'rejuvenation' occurred, would seem to indicate that owing to unfavorable environmental conditions the race of *Oxytricha* was in an unhealthy state which resulted in a series of abortive attempts to reorganize itself, the final one of which was successful.

Popoff ('07) found in the culture of *Stylonychia mytilus*, which he bred for three and one-half months, that after three periods of depression had occurred the race finally became extinct during a period of "sehr tiefe Depression." Popoff does not make a distinction between these various depressions, except in regard to their intensity. He records the fact that at these times characteristic nuclear changes as well as a tendency to conjugation were in evidence though both were most pronounced during the very deep depression. Again, in a culture of *Paramaecium caudatum*, the data from which he does not present in as much detail, he found essentially similar depressions and nuclear metamorphoses. He identifies the deepest periods of depression with those described by Maupas as "dégénérescence sénile" by Hertwig as "physiologischer Tod," and by Čalkins as "depression periods."

In a later study Popoff ('09) stated that he was able to produce, by treatment with various chemical reagents, identical variations in the nuclear apparatus of cultures of *Stylonychia mytilus* and *Paramaecium caudatum*, which in turn he considered similar to those observed by Kasanzeff in starved Infusoria. In other words, Popoff concluded that the nuclear phenomena in all his depression periods are exactly the same as those induced by starvation, chemical stimuli, etc., and further that they cannot be distinguished from those which occur at the onset of normal conjugation. In text figure 21 (Popoff's fig. 25, pl. 2) is shown an animal with two micronuclei in mitosis from a culture of *Paramaecium caudatum* which Popoff had treated with ammonium. This specimen we would interpret as an early 'reduction' division in the reorganization process under discussion.

We have found that it is possible to retard or hasten the occurrence of the process by the character of the culture medium; for example, it may occur a few days earlier in animals not supplied daily with fresh culture fluid than in the regular lines. Consequently we can readily believe that treatment with the reagents employed by Popoff would influence its onset. Popoff figures specimens of *Stylonychia mytilus* in periods of depression and also after chemical treatment which he interprets as showing



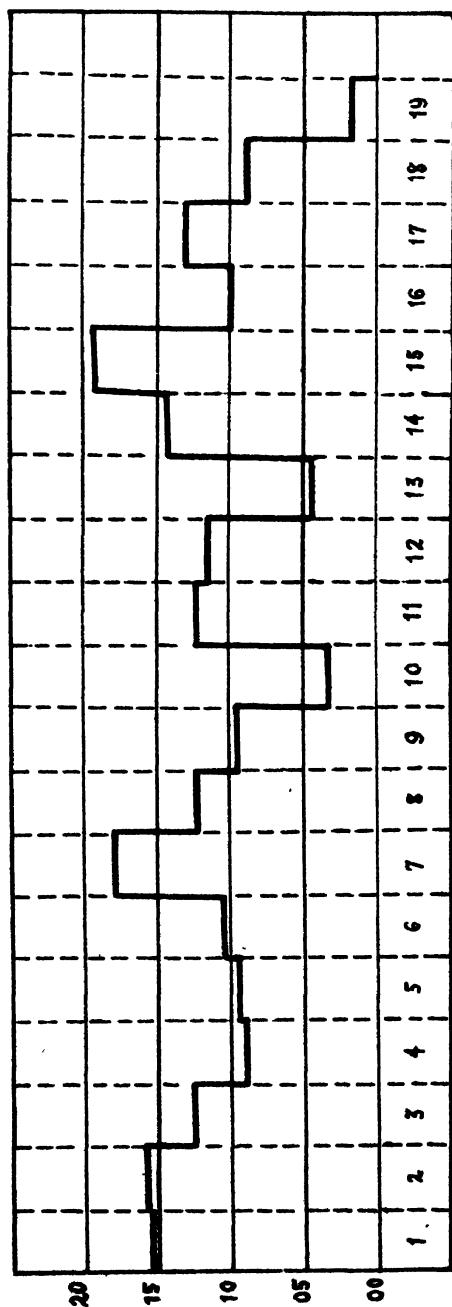
Text figure 21

the multiplication of micronuclei until there are a dozen or more in a cell. Although it seems clear that these represent stages in the process, it is impossible from his figures to be sure that many of the bodies which he interprets as micronuclei are not chromatin bodies which have arisen from the macronucleus as occurs in *Paramecium aurelia*. However, Popoff's paper shows that he recognizes a general similarity of the so-called depression phenomena and the cytological changes incident to the early stages of conjugation. Our data, secured by a daily cytological study of pedigreed animals, throw light upon Popoff's isolated stages and indicate with considerable certainty that they are stages in the sequence of normal nuclear changes in the process which closely parallel conjugation both in morphological and physiological features.

Gregory ('09) in a study of the life history of *Tillina magna* points out that " . . . the curve which represents the general vitality of the protoplasm shows the normal rhythmic fluctuations observed by Woodruff," and in an analysis of the

data secured by Popoff in his study of the life history of *Stylonychia mytilus* shows that "if the curve of *Stylonychia* is plotted from average records of five and ten-day periods, it will be found to correspond to the curves for *Paramecium*, *Oxytricha* and *Tillina*, each showing the rhythmic periods of high and low vitality." We reproduce here (text fig. 22) Gregory's curve plotted from Popoff's data, with the depression periods indicated. This curve is obviously strikingly similar to the ones which we give in the present paper to show the relation between the process and the rhythms. It is to be noted that at the low points between Popoff's 'rhythms,' just as at the low points between our rhythms, nuclear changes occurred. This agreement, taken in conjunction with the fact that Popoff's animals show evidence of the process we describe, probably makes it now safe to state that Popoff's small depression periods are rhythms. Such being the case, we are in a position to identify with more certainty the *minor depression periods of Hertwig and Calkins as rhythms also*.

When one considers the diverse culture methods used by the various authors and that merely cells selected at relatively long intervals and practically at random were studied by them, it is remarkable that so many indications of the process may be gleaned from their data. This affords additional evidence of the general occurrence of this phenomenon in Infusoria. We do not desire, however, to deny that abnormal cytoplasmic and nuclear conditions can and do occur in infusorian cultures, or to seem to attempt to interpret as stages of the process all the so-called degeneration stages figured by various authors. Now and then an animal in the culture of *Paramecium* under consideration has failed to divide for several days and has finally died without signs of the process. A study of such animals has shown what are certainly abnormal conditions of cytoplasm and nucleus. The cytoplasm appears "diluted" and vacuolated, the pellicle has a characteristic glistening effect, and the cell-contents show more acidity than normal. The nucleus may assume various atypical forms. Such a condition may arise in various ways, but the usual one is the contamination of the medium with a



Text fig. 22 Complete history of *Stylonychia*. The curve is plotted from the records of Popoff. Rate of division averaged for five-day periods. Periods 4, 10, 13, 16 are the "deep depression" periods of Popoff. (From Gregory, 1909.)

deleterious bacterial strain. Some types of bacteria produce unhealthy conditions which are readily recognizable when one has conducted a culture for a long time and consequently, when such appear, they can be eliminated. Now and then, however, a culture becomes contaminated with a form which does not produce a characteristic change in the medium and therefore it is not noticed. As a result, at the daily isolations, the fresh medium becomes infected. This was the case with Line VI of Sub-culture IE, as already described (cf. p. 467). The Infusoria, then, can and do degenerate and die as a result of unfavorable conditions, but we wish to reiterate that even under such conditions the cell sometimes attempts to restore its normal equilibrium by the inherent reorganization phenomenon.

It is clear that the cycle emphasized by Maupas, Calkins and others is merely a phantom which has continually receded as each successive investigator has approached the problem with improved culture methods until it has vanished with Woodruff's race of (so far) 4500 generations. What remains then is the rhythm and in the light of the present study, which demonstrates the underlying cytological phenomena of which it is an outward physiological expression, the whole problem takes on a new aspect. The cell automatically reorganizes itself periodically by a process which, in its main features, simulates conjugation—but without a contribution of nuclear material from another cell. Therefore it is evident (as has been shown by this culture) that the formation of a syncaryon, whose components are derived from two cells, is not necessary for the continued life of the cell—it has an internal regulating phenomenon which is entirely adequate to keep it indefinitely in a perfectly normal condition.

VIII. ENDOMIXIS AND ITS RELATION TO CONJUGATION

It is necessary now to consider the significance of the reorganization process in relation to conjugation in which the formation of a syncaryon results in a union of chromatin from two distinct animals.

It has been shown in the main pedigreed culture of *Paramecium aurelia* that at intervals throughout the seven years (to date) of its existence the process has occurred. It has also been shown that when a mass culture of animals from this race was under just the proper conditions, typical conjugation occurred. *Thus it is proved that both the reorganization process and conjugation are potentialities of the animals of the same race*—and therefore there is no evidence for the view of Calkins ('13) that conjugating and non-conjugating races of *Paramecium* exist (cf. p. 429), or that "apparently some paramecia are potential germ cells, others are not." The cell clearly has two methods of reorganization which, so far as the evidence at hand indicates, may be employed indiscriminately. One is conjugation, the other is the reorganization process.

The chief morphological changes incident to conjugation and the process may be contrasted as follows:

In each case the old macronucleus is dissolved in the cytoplasm and micronuclear reduplication occurs until eight are present in the cell;

In conjugation one of these micronuclei persists and by an *extra division* forms the stationary and migratory nucleus. This division is directly preparatory for the accession of foreign chromatin. After the mutual interchange of the reduced micronuclei is effected, a syncaryon is formed. The latter gives rise to the new nuclear apparatus;

In the reorganization process, on the other hand, one or two micronuclei persist (cf. p. 446), from which the new nuclear apparatus arises.

After conjugation the reorganized cell has a new macronuclear and micronuclear apparatus composed of combined material from the two conjugants;

After the reorganization process the reorganized cell has a new macronuclear and micronuclear apparatus composed of material from its own micronuclei.

In a word—the *essential morphological difference is the entire absence of the introduction of foreign nuclear (and cytoplasmic) material into the cell during the reorganization process.*

Since the process results in the dissemination of the material from the old macronucleus and the so-called reduction micronuclei in the cell, it gives the opportunity for a rearrangement of the molecular constitution of the cell. This involves a more profound intermingling of nuclear and cytoplasmic substances than is possible during the typical vegetative life of the cell. *Since this intermingling occurs within a cell we term this reorganization process endomixis.* Endomixis is followed by a slight acceleration of cell phenomena and a new rhythm is initiated.

Similarly, since the consummation of conjugation also results in the dissemination of the old macronucleus and of the 'reduction' micronuclei in the cell it affords the opportunity for a rearrangement of the chemical constituents of the cell. But, further, the exconjugant is supplied with a new nuclear apparatus presumably derived equally from each conjugant. Thus here the introduction of foreign chromatin and possibly cytoplasmic material affords still greater opportunity for chemical rearrangement. Therefore conjugation is a caryomictic phenomenon whereas endomixis, characterized by the non-formation of a syncaryon, is an apocaryomictic process.

The general physiological effect, however, of conjugation in Infusoria is not demonstrated conclusively, though Calkins offers considerable evidence which indicates that, after its consummation, all the processes of the cell, including reproduction, proceed with greater vigor, thus substantiating the view of Bütschli, Maupas, Hertwig and others. Jennings' results on the other hand afford no support for this conclusion.

Without considering this debated question, however, it is evident from this culture that syngamy in *Paramecium* is not necessary, under proper environmental conditions, for the continued life of the race, and therefore that the formation of a syncaryon, composed of material partly derived from another cell, is not a *sine qua non* for a proper continuation of all the vital activities of the cell. We would therefore put emphasis on molecular rearrangement as the result common both to endomixis and to conjugation. Since accelerated vital activities follow this chemical change afforded by endomixis, it seems clear

that a similar condition follows conjugation, and therefore that one aspect of conjugation (though it is unnecessary for the life of the race under proper environmental conditions) is dynamic.

Certain observations which we have made on mass cultures of *Paramaecium aurelia* indicate that endomixis and conjugation may occur simultaneously in different animals of the same culture, thus strongly suggesting that the same *general* conditions lead to both phenomena—one animal meeting the conditions one way and another by the other, and that both phenomena fill essentially the same place in the economy of life of *Paramaecium aurelia*. Therefore it is evident that the vegetative aspect of the life history of *Paramaecium* is periodically interrupted by periods in which a dynamic reorganization occurs either by endomixis or by conjugation.

IX. ENDOMIXIS AND ITS RELATION TO PARTHENOGENESIS

One might at first glance consider endomixis as a case of parthenogenesis, owing to certain similarities of the two phenomena. In parthenogenesis the new individual arises from one nucleus which is not a syncaryon, i.e., the fusion of completely independent nuclei. In endomixis the new nuclear apparatus is of micronuclear origin without the formation of a syncaryon. Both parthenogenesis and endomixis therefore are apocaryomictic phenomena which must be contrasted with caryomictic phenomena such as conjugation, copulation and, to a certain extent, autogamy. The introduction of chromatin belonging either to another individual or to another nucleus of the same individual gives an opportunity for an extensive rearrangement of the nuclear substance which cannot be effected to such a high degree in either parthenogenesis or endomixis.

Although both parthenogenesis and endomixis are followed by *Entwicklungsarregung* they afford some features which are not identical. In parthenogenesis there is a chromatin reduction which occurs and is compensated for either in the egg itself or in some later period of the life cycle of the race.

Following the definition of Winkler ('08, p. 303), parthenogenesis may be considered as the "apomiktische Entstehung eines Sporophyten aus einem Ei," it being of minor importance whether the reduced or unreduced number of chromosome is present. Strasburger ('07, p. 170) on the other hand puts the greatest weight on the chromosome number and applies the term parthenogenesis only to cases in which an egg with the haploid number of chromosomes undergoes *Entwicklungserregung*. According to the view of Strasburger, the reorganization process in *Paramecium* cannot be termed parthenogenesis. In endomixis there is no evidence of a chromosome reduction. This race has existed normally without apparent change for over seven years, during which time it has undoubtedly undergone endomixis nearly one hundred times and thus it is clear that a chromatin reduction, involving a loss of specific chromatin or chromosomes and therefore according to the prevailing view a loss of hereditary units or gene material, cannot have occurred in endomixis. Further, the nuclear material from which the new nuclear apparatus arises in endomixis cannot be considered as a gamete, because the third micronuclear division which forms the two gametes in *Paramecium aurelia* does not occur in endomixis. The eight "reduction" micronuclei are comparable to gametocytes, the *paramecium* individual being a gamont before it starts to form the eight so-called reduction micronuclei (Lühe, Hartmann). So according to the definition of Winkler it seems impossible to consider endomixis as parthenogenesis in *sensu stricto*.

We therefore have employed a new term 'endomixis' for the reorganization process in *Paramecium*, in preference to parthenogenesis which Hertwig applied when he incidentally noted some isolated stages of the nuclear phenomena which we have elucidated. The stages observed by Hertwig were insufficient to give a clue to the sequence of cytological changes—this being necessary for a realization of the importance and proper classification of the reorganization process.

If subsequent research should show (which from our work seems highly improbable) that a third micronuclear division, involving a chromosome reduction, does occur in the descending

phase of the process which we term endomixis, it is obvious that the phenomenon would still fall under endomixis and not under parthenogenesis since the latter term is, according to our interpretation, restricted to cases in which gametes undergo *Entwicklungserregung*. The only way to justify the application of the term parthenogenesis is *sensu stricto* to the phenomenon which we call endomixis would be by proving that in the latter process a reduction of the number of chromosomes actually takes place in a micronucleus which can be considered as the equivalent of a gamete, and that for this reduction there is a compensation.

However, if one is inclined to employ the term parthenogenesis in a very broad sense to include all cases of *Entwicklerregung* without regard either to the character of the cell (egg, gametocyte, gamont, somatic cell) in which they occur, or to haploid or diploid condition of the chromosomes, then endomixis is a new type of parthenogenesis. It is always difficult to relate properly a newly discovered phenomenon to analogous processes. In the present instance this is doubly difficult owing to the fact that in the protista the formation of a typical macrogamete closely analogous with that of metazoa and metaphyta is the exception rather than the rule. It is probable that future study of other *Protista* (e.g., bacteria, amoeba, trypanosomes, plasmodia, etc.) will reveal nuclear reorganization processes which will make certain the proper place of endomixis among the already complicated apocaryomictic phenomena.

X. ENDOMIXIS AND ITS RELATIONS TO VARIATION, HEREDITY AND THE SIGNIFICANCE OF CONJUGATION

We deem it inadvisable in the present paper—which is essentially a description of cytological and physiological observations—to enter into an extended discussion of the wide theoretical bearings of endomixis on the problems of variation, heredity and amphimixis. It is important, however, to consider briefly certain points which are obviously suggested by the observations described. This may be facilitated by a rapid survey of the facts which are to be interpreted.

1. *Paramecium aurelia* can reproduce indefinitely without conjugation under favorable environmental conditions. The so-called life cycle is non-existent.

2. Minor periodic fluctuations (rhythms) occur in the rate of reproduction.

3. The rhythms are the obvious physiological expression of periodic internal phenomena.

4. The internal nuclear phenomena comprise the formation of a complete new nuclear apparatus of micronuclear origin. This process we term 'endomixis.'

5. The essential cytological difference between endomixis and conjugation is the absence of the third division which in conjugation forms the stationary and migratory micronuclei, and of necessity the non-formation of a syncaryon.

Now it is important to emphasize again that this race of *Paramecium aurelia* has existed (so far) for over seven years and through more than 4500 generations in a perfectly normal manner without conjugation, during which time it has undergone endomixis frequently, undoubtedly on the average once each month. Further, conjugation has been successfully consummated by animals from this culture and therefore *both* endomixis and conjugation are normal phenomena in the life of this same race.

It seems clear then that this culture offers strong *physiological* evidence against the interpretation of either of the first two so-called reducing micronuclear divisions as actually being a *chromosome reducing* division. Since the nuclear changes in endomixis and conjugation are fundamentally the same except for the absence of the third micronuclear division in the former, it is justifiable to regard this third micronuclear division which occurs in conjugation as the one directly preparatory, from the standpoint of chromosome reduction and sexual phenomena, for the imminent accession of foreign chromatin in the form of the migratory micronucleus of the other conjugant.

Endomixis, involving as it does the disintegration and absorption of the old macronuclear and micronuclear material, affords the opportunity for molecular rearrangement and therefore may afford the opportunity for the origin of variations within a pure

line of *Paramecium*. Jennings in his extensive studies on this organism has been able to discover only variations about the mean in pure lines without conjugation. These variations about the mean, we believe, are probably brought about, in part at least, by endomixis because, although there is no opportunity in this process for an introduction of foreign nuclear material, nevertheless there is an opportunity for its rearrangement. But Jennings admits that "There remains the possibility that heritable variations of a totally different (lesser) order of magnitude may arise during vegetative reproduction" ('13, p. 355). There is no evidence from the race of *Paramecium* which is the basis of our studies that variations in morphological features or in the division rate have occurred during its long cultivation which have been inherited—the animals of the 4500th generation being apparently in all ways similar to those of the early generations—but we wish to point out that, a priori, endomixis affords a field for the origin of such variations. It is conceivable that 'heritable' variations may result from some rare recombinations in endomixis. Biometrical studies of animals in isolated lines subsequent to endomixis are highly desirable.

In higher organisms fertilization has a dynamic as well as a hereditary rôle, and that these may be separated is amply attested by so-called artificial parthenogenesis. In the conjugation of *Paramecium*, Calkins, among recent investigators, has put emphasis on the dynamic aspect, though admitting the probability that conjugation is a source of variation; while Jennings definitely states that "There is no evidence that conjugation in the infusoria increases the reproductive power or rejuvenates the organism physiologically in any way" and puts all the emphasis on the side of variation and heredity.

Endomixis does initiate a new rhythm in the life history of *Paramecium*, i.e., a period of increased metabolic activity and therefore of reproductive activity, and since its fundamental morphological features are almost identical with those preliminary to the formation of the stationary and migratory micronuclei in conjugation, it lends strong support to the view that the dynamic aspect of conjugation is not absent.

From the life history of this race of *Paramecium aurelia* we lean toward the view that both endomixis and conjugation in the infusorian, as fertilization in higher forms, have a two-fold significance—both afford the opportunity for molecular rearrangement which leads to increased physiological activity, and the opportunity for variation. The life of the *paramecium* race can proceed indefinitely with endomixis under favorable environmental conditions—conjugation being unnecessary. “Senile degeneration” and “physiological death” are not the inevitable result of continued reproduction without conjugation—the cell has an internal regulatory phenomenon, endomixis, which is self-sufficient for the indefinite life of the race.

From whatever standpoint one views the wealth of data which has been secured during recent years in regard to the problems of variation, heredity, and the significance of conjugation in Protista, it is clear that hereafter, in the interpretation of previous and the prosecution of subsequent studies, due weight must be accorded to endomixis.

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EXPLANATION OF PLATES

All the figures represent specimens of *Paramecium aurelia*, except figure 34. The animals figured in red were fixed with sublimate-acetic and stained with Ranvier's picrocarmine; those figured in blue were fixed in Schaudinn's sublimate-alcohol and stained with Delafield's hematoxylin. The drawings were made from total preparations, with Abbe camera lucida, Zeiss homogeneous immersion 2 mm. and compensating ocular 12, with drawing board level with stage of microscope. Magnification, about 1500 diameters. Reduction for plates, one-half, which has obscured some micronuclear details.

PLATE 1

EXPLANATION OF FIGURES

Descending Phase

1 Main Culture I, Line b, 424th generation, April 11, 1908. Normal animal in the period when the reorganization process is not in progress.

2 Subculture IE, Line VI, 4020th generation, October 27, 1913. Normal animal in the period when the reorganization process is not in progress.

3 Subculture IE, Line VII, 4236th generation, February 21, 1914. Animal just before the beginning of the process in the 4238th generation. Macronucleus with coarse granules; two micronuclei which have shifted from their ordinary position close together at one side of the macronucleus.

4 Subculture IE, Line IV, 4044th generation, November 10, 1913. Small projections from the macronucleus; two resting micronuclei, and several vacuoles containing bacteria.

5 Subculture IE, Line VI, 4094th generation, December 9, 1913. Large projections from the macronucleus, which is coarsely granular. Two micronuclei shifted from their typical position.

6 Main culture I, Line c, 452d generation, April 30, 1908. Two chromatin bodies which have been ejected from the partially membranous macronucleus; two micronuclei shifted from their typical position.

7 Subculture IE, Line VI, 4182d generation, January 24, 1914. Animal in process of division during the descending phase of the process. One chromatin body, numerous vacuoles, four division micronuclei and the remnant of a division spindle.

8 Subculture IE, Line V, 4023d generation, October 30, 1913. Several chromatin bodies, some within and some which have passed out from the macronucleus. Two isolated micronuclei showing indications of division.

9 Subculture IE, Line II, 4069th generation, December 2, 1913. Macronucleus still further resolved into chromatin bodies; micronuclei are obscured by the macronucleus. Numerous vacuoles present.

10 Subculture IE, Line VI, 4130th generation, December 25, 1913. Numerous chromatin bodies the position of which in the cell indicates the effect of cytoplasmic currents. Macronucleus partially membranous. Animal started to effect the process which however was actually accomplished in this line in the 4140th generation. One micronucleus visible.

11 Subculture IE, Line IIIa, 4271st generation, March 12, 1914. Almost completely disintegrated macronucleus. Two micronuclei dividing to form four 'reduction' micronuclei; an irregular division in a third micronucleus.

12 Subculture IE, Line VII, 4314th generation, April 4, 1914. Macronucleus nearly devoid of chromatin; numerous chromatin bodies; three micronuclei dividing to form six 'reduction' micronuclei.

13 Culture B, Berlin race, October 6, 1913 (see page 473). Numerous chromatin bodies; six so-called reduction micronuclei. Climax of process.

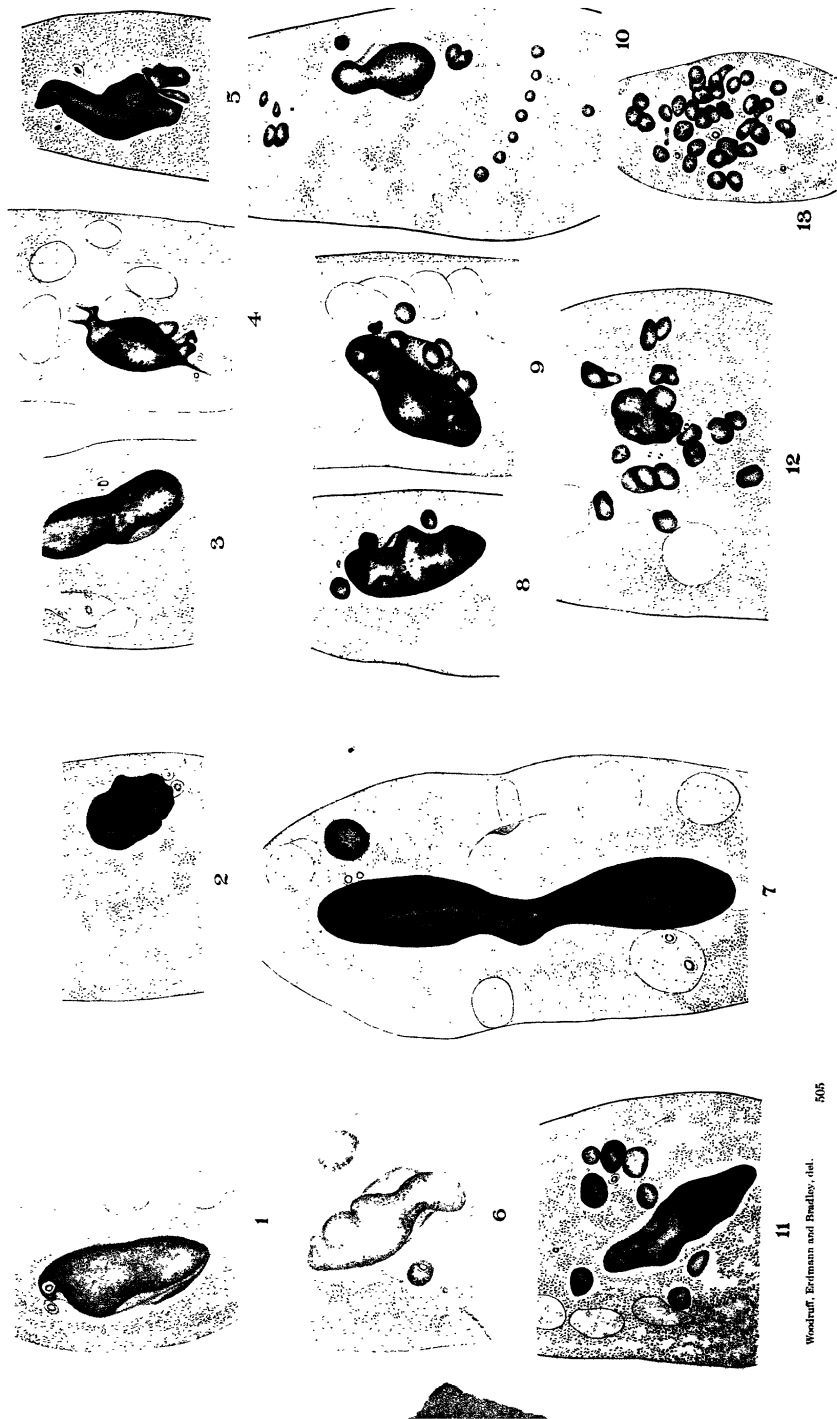


PLATE 2

EXPLANATION OF FIGURES

Climax

14 Main culture I, Line c, 426th generation, April 9, 1908. Old macronucleus merely in the form of a membrane from which the numerous chromatin bodies have been ejected and are free in the cytoplasm. Eight so-called reduction micronuclei two of which are lying isolated; the others in groups of three.

15 Main culture, Line b, 1498 to 1499th generation, December 12, 1909. A division stage at the climax of the process. Numerous chromatin bodies in each sister cell. One shows a membranous remnant of the old macronucleus. Each has only one micronucleus.

16 Mass culture started from main Culture I, October 12, 1913 (see page 435). One cell is in same stage as both cells in figure 15. The other cell shows the formation of the macronuclear anlagen, several chromatin bodies and two micronuclei.

17, 18 and 19 Culture B, Berlin race, October 6, 1913 (see page 473). Three animals illustrating the details of the formation of the macronuclear anlagen. Figure 17 shows two micronuclei, one macronuclear anlage completed and the other macronuclear anlage just beginning to be evolved from a micronucleus. Figure 18 shows two micronuclei and two completely formed macronuclear anlagen. Figure 19 shows the same except that the micronuclei have divided for the next cell division. In these three figures the chromatin bodies have been omitted. The cells have been counterstained with eosin.

20 Subculture IE, Line VIa, 4084th generation, December 4, 1913. Animal shows two macronuclear anlagen which have lost their initial form. Chromatin bodies beginning to disintegrate. Two micronuclei.

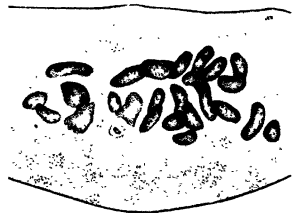
21 Subculture IE, Line VI, 4107th generation, December 16, 1913. The same stage as shown in figure 20, except that one of the micronuclei has completed division and the other is in process of division.

22 Subculture IE, Line III, 4063d generation, November 23, 1913. Essentially the same stage as former but showing both micronuclei in division.

23 Main culture I, Line c, 1755th generation, May 1, 1910. Animal figured to show that the complete reorganization of the cell occurred in the same manner at a relatively early period in the history of the main race.

24 Culture B, Berlin race, March 15, 1913. Animal figured to show that the complete reorganization occurred in an animal from a *different* race. Only one micronucleus is evident, the other being obscured by the chromatin bodies.

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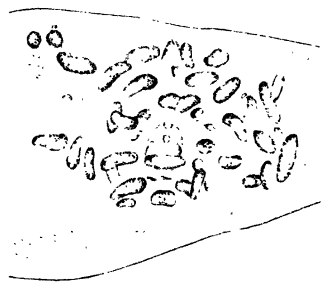
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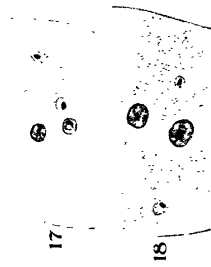
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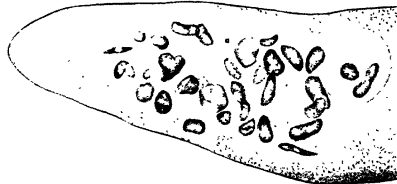
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PLATE 3

EXPLANATION OF FIGURES

Ascending Phase (Except figures 31 to 34)

25 Subculture IE, Line VIb, 4174th generation, January 18, 1914. Animal after the first cell division. One macronuclear anlage. Some chromatin bodies in process of disintegration and enclosed by vacuoles. One micronucleus has divided and the other is in process of division for the ensuing cell division.

26 Subculture IE, Line VI, 4185th generation, January 25, 1914. A characteristic stage of the ascending phase of the process. New macronucleus with fine chromatin granules. Two micronuclei present in cell but one near end omitted from drawing. Chromatin bodies disintegrating. Cf. sister cells, text fig. 8.

27 Subculture IE, Line IIb, 4313th generation, April 3, 1914. New macronucleus, two micronuclei, four chromatin bodies and the cloud-like chromatin remnants of the others.

28 Main culture I, Line d, 1432 to 1433d generation, November 7, 1909. Young animals just after division during the ascending phase of the process. Micronuclei in each show various stages of division for the following cell division. The cell division just being completed is the fifth in about twenty-four hours (see page 456). A few chromatin bodies still present.

29 Subculture IE, Line VI, 4187th generation, February 27, 1914. New macronucleus, two micronuclei and three chromatin bodies.

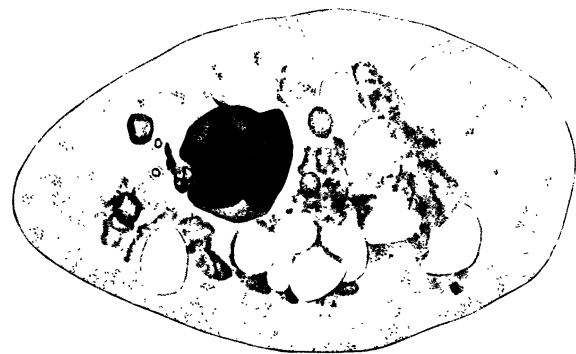
30 Main culture I, Line b, 1201st generation, June 7, 1909. New macronucleus, two micronuclei and two remaining chromatin bodies one of which is under the macronucleus.

31 Mass culture seeded from main Culture I. An epidemic of conjugation in progress. Animal with micronuclei in process of division.

32 Pair of *conjugants*, showing first micronuclear division after formation of the syncaryon (cf. page 429 and page 453).

33 An *exconjugant* from isolated conjugating pair. First vegetative division completed. Macronuclear anlagen assuming typical macronuclear form. Note finely granular condition of chromatin typical of new macronucleus. Two micronuclei are present. Five food vacuoles with bacteria and promiscuous culture material in which conjugation was secured.

34 *Paramecium caudatum*, Culture Y, Line 2, 139th generation, April 29, 1914. Descending phase of the process showing the formation of the chromatin bodies in this species. The one characteristic micronucleus of *caudatum* has divided. Only optical section of micronuclei is drawn.



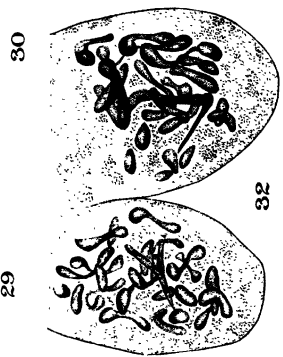
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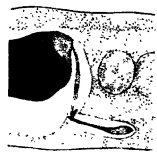
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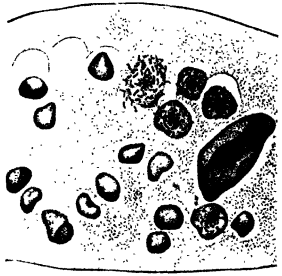


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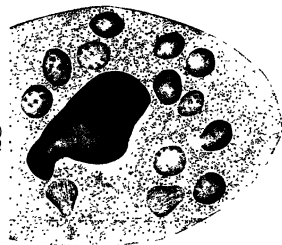
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PLATE 4

EXPLANATION OF FIGURES

Additional Stages

35 Subculture IE, Line VIb, 4092d generation, December 12, 1913. Typical form of macronuclear disintegration. Macronucleus small. Numerous chromatin bodies. Three micronuclei present.

36 Subculture IE, Line VI, 4102d generation, December 14, 1913. Typical form of macronuclear disintegration. Macronucleus of typical size, practically devoid of chromatin. Numerous chromatin bodies. Four reduction micronuclei. (cf. figs. 11 and 12). A₂, text figure 4, is sister cell.

37 Subculture IE, Line VIa, 4087th generation, December 6, 1913. An atypical form of macronuclear disintegration, slightly resembling the ribbon-like formation characteristic of conjugation. Beginning of micronuclear reduction.

38 Mass culture started from main Culture I, October 12, 1913. Transverse section through an animal at the climax. Seven chromatin bodies, two micronuclei in a cytoplasmic layer are visible. Stained with safranin.

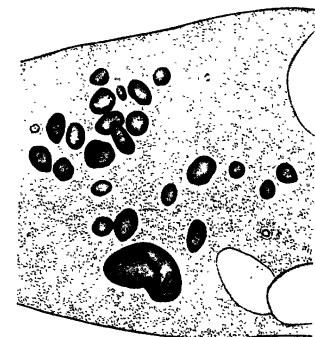
39 Mass culture started from main Culture I, October 12, 1913. The posterior cell is in the same stage as both cells in figure 15. The anterior cell, after the formation of the macronuclear anlagen, has effected one more micronuclear division than the animal in figure 16a. The food vacuoles present contain remnants of material from hay infusion medium.

40 Subculture IE, Line VII, 4355th generation, April 24, 1914. Stage at the end of the climax after the cell division characteristic of this period. Half the products of the next micronuclear division will become the two macronuclear anlagen.

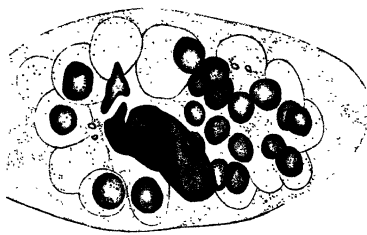
41 and 42 Subculture IE, Line VIa, 4436th and 4437th generations, June 12, and 14, 1914. Cell 4436 has completed the cell division in the climax. Numerous chromatin bodies and one micronucleus. The sister cell in the 4436th generation, which kept the line alive, formed the macronuclear anlagen and by a division in about forty-eight hours distributed these to each of the two cells of the 4437th generation. One of these cells is shown in figure 42. Numerous chromatin bodies, one macronuclear anlage and four micronuclei. The micronuclear division indicates the quick succession of cell divisions characteristic of the ascending phase.

43 Subculture IE, Line VIq, 4437th generation, June 13, 1914. Animal after the first division in the ascending phase. New macronucleus with condensed chromatin. Three micronuclei.

44 Mass culture from main Culture I. Shows ribbon-like formation of macronucleus in a *conjugating* animal (cf. figs. 32 and 37). Eight reduction micronuclei, three of which are drawn.



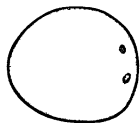
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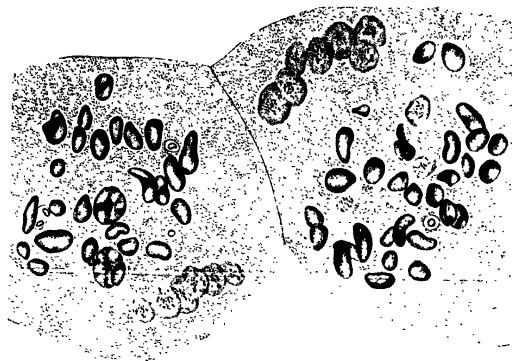
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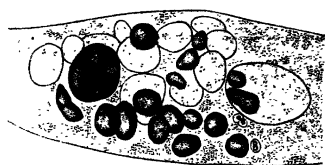
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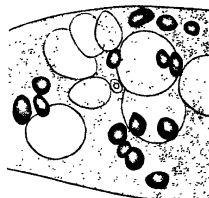
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THE REACTION OF EMBRYONIC CELLS TO SOLID STRUCTURES

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FOURTEEN FIGURES

It is generally recognized that the movement of cells and cell masses is an essential factor in morphogenesis.¹ The most obvious movements concerned in the development of the Metazoa are those of cell aggregates, but these are often complicated by their association with growth or increase in mass. On the other hand, the movements of single cells, involving growth only to a very slight extent, are less complex and, at the same time, with our present methods of tissue culture, are readily amenable to observation and experiment. The mechanism of this movement is the streaming of the cell protoplasm, and the ontogenetic results depend upon the physical properties of the protoplasm itself and the stimuli which act upon it.

The importance of such factors in development was fully recognized twenty years ago by Roux² in his experimental study of the behavior of isolated cells of the frog's blastula, and by Herbst³ in his 'Reizphysiologie.' Even before this Loeb⁴ had considered the tropisms of cells, and had shown that the arrangement assumed by certain chromatophores in the fish embryo is dependent upon a stimulus emanating from the circulating blood. Somewhat later Driesch⁵ also took up the question and found a

¹ The various types of movement occurring in ontogeny have been carefully classified by Davenport in his "Preliminary catalogue of the processes concerned in ontogeny." *Bull. Mus. Comp. Zool. Harvard Coll.*, vol. 27, 1896.

² Cytotropismus. *Arch. f. Entw. Mech.*, Bd. 1, 1894; Bd. 3, 1896.

³ *Biol. Zentralbl.*, Bd. 14 and 15, 1894, 1895. Also *Formative Reize*, Leipzig, 1901.

⁴ *Jour. Morph.*, vol. 8, 1893.

⁵ *Arch. f. Entw. Mech.*, Bd. 3, 1896.

striking case of a cellular tropism (taktische Reizbarkeit) in the behavior of the mesenchyme cells of the sea urchin embryo. It must likewise not be forgotten that as early as 1892 Ramón y Cajal had stated his theory of chemotaxis to account for the outgrowth of the nerve fiber.⁶ Inexplicable as it may seem, very little of a definite nature has been added to our knowledge of this field since the period just cited.

In my work on the development of peripheral nerves⁷ it was definitely shown in confirmation of Cajal's theory, that the active factor in the spinning of the nerve fiber is a small mass of amoeboid protoplasm at the end of a cell process. The formation of long fibers by means of this mechanism was found to be a characteristic of embryonic nervous tissue, but other somewhat different forms of activity of the same general nature were seen in all cells of the frog embryo when cultivated in clotted lymph.⁸

No rigorous proof of the reaction of moving cells to definite stimuli was given in the course of these experiments. One of the most striking circumstances noted, however, was that when the lymph clotted firmly the movement was nearly always active, while in the cases in which the medium remained fluid only rounded cells were seen and these failed to undergo characteristic changes of form and locomotion. These observations led to the hypothesis that the cells were positively stereotropic and therefore unable to leave the solid masses to move out into purely fluid media. The work of Burrows with tissues of the chick embryo lent support to this hypothesis and it has since received further confirmation from other sources.⁹ Prior to this L. Loeb,¹⁰

⁶ La rétine des vertébrés. La Cellule, T. 9, 1892.

⁷ Anat. Rec., vol. 1, 1907; Jour. Exp. Zool., vol. 9, 1910.

⁸ Cells with processes of hyaline protoplasm were seen by Roux in the course of his work on cytotropism. The pseudopodia were described by him as occurring on cells that had become attached to the bottom. In one passage Roux states (op. cit., p. 163) that it is doubtful if any cytotropic movements take place without the solid base.

⁹ Burrows, Jour. Exp. Zool., vol. 10, 1911; Carrel and Burrows, Jour. Exp. Med., vol. 14, 1911; Holmes, Univ. California Pub., Zoölogy, vol. 11, 1913.

¹⁰ Arch. f. Entw. Mech., Bd. 6, 1897; Bd. 13, 1902; Zeitschr. f. Krebsforschung, Bd. 5, 1907.

from his studies on the growth of epithelium and carcinoma, was likewise led to the view that these tissues are stereotropic.

Nevertheless, none of these cases established the occurrence of this phenomenon beyond doubt. It seemed to be only a probable explanation of certain scattered facts. The experiments described in the present paper were designed to put the hypothesis to a more rigorous test.

Examples of stereotropism (thigmotaxis) have long been known in plants and there is a wide range of reactions in animals that may be gathered under this head, some of which have been considered by J. Loeb¹¹ and by Verworn.¹² These reactions, are however carried out mostly by complex mechanisms and have but little direct resemblance to the cellular phenomena here considered. The best known case of positive stereotropism in single cells is that of the spermatozoa of *Periplaneta*, originally described by Dewitz,¹³ who called attention to its significance in the act of fertilization. Jennings's¹⁴ description of the movements of amoeba indicate that here also reaction to solids plays an important part in locomotion.

METHOD OF EXPERIMENTATION

The plan of experimentation in the present work consisted in varying the three principal factors involved in the cultivation of tissues, viz., the tissue itself, the fluid medium, and the solid support. The results have shown that all three of these factors have some determining relation to the movement observed. Each tissue has certain characteristics, as seen especially in the differences manifested by epithelium, connective tissue and nervous tissue; the constitution of the fluid medium has a marked effect upon the vigor of the movement; the solid support influences the form and arrangement assumed by the moving cells, and its

¹¹ *Heliotropism der Tiere*. Würzburg, 1889; *Studies in general physiology*, Chicago, 1905; *Dynamics of living matter*, New York, 1906; Winterstein, *Vergleichende Physiologie*, Bd. 4, article on Tropisms.

¹² *Bewegung der lebendigen Substanz*, Jena 1892; *Allgemeine Physiologie*.

¹³ *Pflüger's Archiv.*, Bd. 38, 1886.

¹⁴ *Behavior of the lower organisms*. New York, 1906.

indispensability is shown by the fact that no movement takes place in its absence.

The first experiments were made with tissues from embryos of *Rana palustris* in the stage just after closure of the medullary folds. These were followed by a series of experiments with the tissues of chick embryos of various ages.¹⁵ The fluid media used were physiological sodium chloride, Locke's and Ringer's solutions in varying degrees of concentration, blood plasma and serum of the frog and the chicken respectively. The means used to support the planted tissues were the fibrin net work of the clotted plasma, spider web, the surface of the cover-glass, and, in some accidental cases, the surface film of the fluid drop. Control experiments were made in large drops of the fluid media, in which the tissue was prevented by the size of the drop from touching the cover-slip.

At the time the experiments with frog tissues were made it was not fully realized that the cover-slip might serve to support moving cells, so that in respect to the solid support only cultures upon spider web and in free hanging drops were made. In some of the latter the pieces of tissue came into contact with the cover-slip accidentally and cells began to wander out on the glass.

The technique used was in the main that employed in the study of the development of nerve fibers,¹⁶ together with the modifications introduced by Burrows.¹⁷ Sterilized apparatus and fluids were used throughout. The cultures were all made by the hanging drop method, the cover-glass being placed over the hollow of a deep depression slide, or upon a thin glass ring of about 20 mm. diameter and 2 mm. height and sealed on with vaseline. The frog tissues were kept at room temperature, while those

¹⁵ A preliminary account of the experiments with frog embryo cells was published in *Science*, vol. 34, 1911. The results upon chick tissues have been referred to briefly in several general papers (see *Anat. Rec.*, vol. 6, 1912; *Trans. Am. Cong. Phys. and Surg.*, 1913). The chick experiments were made with the assistance of Mr. (now Dr.) Paul G. Shipley, to whom it gives me much pleasure to express my thanks.

¹⁶ *Jour. Exp. Zool.*, vol. 9, 1910.

¹⁷ *Jour. Am. Med. Assoc.*, vol. 65, 1910; *Jour. Exp. Zool.*, vol. 10, 1911.

from the chick were incubated at about 39°C. The unusual hot weather which lasted during almost the whole period when the experiments with chick tissues were under way rendered unnecessary any precautions to keep the tissues warm during their preparation and examination.

The preparation of the spider web for the experiments was the only innovation in technique that needs special description. It was necessary to have the web tensely spread over a suitable frame so that it would support the drop of fluid from below. For this purpose glass rings were employed, since they were easy to sterilize and well adapted to form the wall of a moist chamber. A number of rings were placed in clean glass aquarium jars and a single spider introduced, which in the course of a day or two spun a web covering the whole bottom of the jar. The rings were then lifted out with forceps and the web cut or torn off around them. The slight roughness of the rim of the glass was sufficient to hold the web tense. The rings with the web upon them were sterilized by dry heat, the web standing a temperature of 150°C. without injury. In making the spider web preparations, the rings were first fastened to the slide with vaseline. A small drop of the culture fluid was then placed upon the web and the tissue afterwards introduced by means of a capillary pipette. It was unfortunately necessary to use very small drops of fluid since the weight of large drops caused the web to sag and touch the bottom of the chamber. The small size of the drops is sufficient to account for the relatively unfavorable results obtained by this method, the tissues usually not growing with very great luxuriance. After mounting the tissue upon the web, a cover-slip was placed over it and sealed on by vaseline. The weight of the cover-slip flattened out the small drop and the tissue was thus in contact both with the cover and the web. In most cases the covers themselves were coated with web so that the tissue was kept between two layers of this material. During the early part of the season there was some difficulty in finding a sufficient number of suitable spiders for the purpose.¹⁸

¹⁸ I am greatly indebted to Prof. A. Petrunkevitch for advice and assistance in collecting this material.

Tiginaria, which may be caught in old tree stumps, proved to be the best adapted of those which were obtainable early in the season, and two large specimens wove enough web for all of the experiments with frog tissues. Later the common grass spider, *Agalena*, was obtained in abundance. This form spins an extremely fine but dense web which is admirably adapted for the purpose. The individual threads of the web are amazingly thin and even under the oil immersion lens they appear as fine lines, the thickness of which can scarcely be measured.

EXPERIMENTS WITH TISSUES OF THE FROG EMBRYO

From this material seventy-one cultures were made in all. They are grouped in several series, of which the first two were of a preliminary nature, having been designed to ascertain what fluid media could be used.

Series I. Only inorganic media were used, as follows: tap water, 0.325 per cent NaCl, Ringer's solution without sugar, Locke's solution half diluted. Solid support was afforded in all cases by spider web. The tissues used were: medullary cord alone, medullary cord with axial mesoderm attached, and ectoderm. Eight preparations showed cell movements. Of the six that gave negative results, three were those which were mounted in tap water; they disintegrated on the second day. Two were in the dilute sodium chloride. One specimen in dilute Locke's solution lived seven days and the others from one to four days.

Series II. In these experiments both inorganic solutions (Ringer and 0.4 per cent NaCl) and defibrinated serum were used. Thirteen pieces were supported on web and eight were put up in hanging drops. None of the latter showed any movement of cells except one in which the drop touched the bottom of the chamber. In this some cell movement took place on the glass. Only one of the cultures in the saline solutions on the web gave positive indications of movement, the others showing no promise from the beginning. Of the nine in serum five were on web and four unsupported. Four of the former showed active movement, one was evidently injured since histolysis of the tissue began

almost immediately. None of the unsupported ones gave positive results.

Series III. This series of twenty-four experiments was designed to test rigorously the influence of the spider web upon the movements of the tissue, and as the preliminary experiments indicated that serum afforded a better medium than the inorganic solutions, the former alone was used. The serum was obtained from three different specimens of *Rana clamitans*, a species different from that of the embryonic tissue isolated. The cultures in this series were made in pairs. The central nervous system with some mesoderm attached was dissected entire out of the embryo in saline solution. It was then divided into two parts, one part being mounted upon web and the other in a plain hanging drop. In some cases the cephalic half of the medullary cord was placed on the web and the caudal half in the plain drop, while in others the order was reversed. The results of this series were entirely convincing. Out of the twelve preparations mounted upon the web eleven showed characteristic wandering of the cells with definite relations to the web fibers (fig. 1). The remaining one was disarranged accidentally the first day and gave no results. Cell movement began in some cases as early as eight hours after explantation and on the day following it was in full swing in all of the cultures. Of the twelve mounted in the large drops none showed any movement of cells except one of the cases in which the drop spread out, allowing the tissue to come into contact with the cover (fig. 3).¹⁹ In this one case six days after the culture was made a number of cells appeared on the cover and later some pigment cells were found. In the large drop preparations the isolated cells, which soon became very numerous,²⁰ always remained rounded (fig. 2), but in spite of considerable disintegration into single cells the main mass of tissue was in every case left intact. That it was alive was

¹⁹ The figure, which is much like the preparation referred to, was drawn from another case.

²⁰ In this respect embryonic frog tissues differ markedly from those of the chick, in which the elements remain closely bound together and the whole mass rounds off its outer surface (see p. 539 and figs. 11 and 14).



Unless otherwise stated figures were drawn from living specimens.

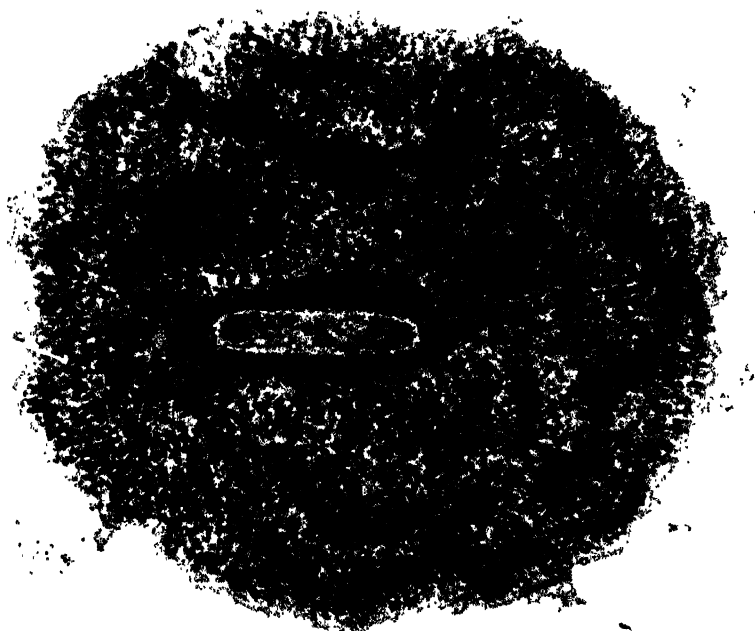
Fig. 1 Experiment S. W. 33. Medullary tube of embryo of *Rana palustris* cultivated 27 hours on spider web in serum from *R. clamitans*. Some cells have moved out in sheets on the cover-slip; others are adapted to the web fibers. $\times 50$.

Fig. 2 Experiment S. W. 34. Medullary tube from same embryo, cultivated 28 hours in large hanging drop of same serum as in figure 1. Considerable disintegration of tissue, but isolated cells are rounded and exhibit no active movements. $\times 50$.

Fig. 3 Experiment S. W. 66. Medullary tube (and notochord) of *palustris* embryo cultivated two days in Locke's solution. The drop spread, leaving tissue in contact with cover. Detached cells are all on the glass. $\times 50$.



2



3

shown by the fact that the cells of the myotomes differentiated into muscle fibers, which after about five days began to contract sporadically, indicating a functionally intact neuro-muscular mechanism. The results of this set of experiments are summarized in table 1.

Series IV. The twelve preparations of this series were put up in the same way as the last except that Locke's solution was used instead of serum. The results were not so conclusive as in the last set, though they presented no contradictory evidence. Only two of the six spider web preparations showed cell movement in any considerable degree, two others were doubtful, while the remaining two gave no promise of activity at any time.

TABLE 1

Experiment III. Tissue in all cases medullary cord and myotomes

NO.	CHARACTER OF PREPARATION	CELL MOVEMENT TOOK PLACE	NERVE FIBERS FORMED	STRIATED MUSCLE FIBERS OBSERVED	MUSCLE TWITCHING TOOK PLACE	DURATION OF OB- SERVATION IN DAYS
31	Web	+				8
32	Round drop			+	+	22
33	Web	+				4
34	Round drop			+		22
35	Web	+				3
36	Spread drop					12
37	Web	+				2
38	Round drop				+	22
39	Web	+				20
40	Round drop				+	8
41	Web	+				6
42	Round drop				+	22
43	Web	+	+			6
44	Spread drop					12
45	Web	+	+		+	22
46	Spread drop					12
47	Web	+				12
48	Round drop				+	22
49	Web	+			+	29
50	Round drop			+	+	22
51	Web ¹					1
52	Spread drop ²	+			+	22
53	Web	+			+	22
54	Spread drop			+		22

¹ Injured.

² A few outwandering cells found on cover.

In the cultures mounted in plain drops the tissue was found in two cases to be in contact with the cover-slip, upon which a few cells wandered out from the main mass (fig. 3). A small number of nerve fibers were seen growing out upon the cover in one case. None of the others showed any cell movement. It is clear from the experiments that the inorganic media are not so favorable for the cultivation of tissues as plasma or serum.²¹

Table 2 gives a summary of the experiments with frog tissues.

The general character of the different types of culture is shown in the figures. Figures 1 and 2 represent two preparations from

TABLE 2
Summary of results of experiments with frog tissues

CHARACTER OF CULTURE	NUMBER OF PREPARATIONS	CELL WANDERING OCCURRED ON SOLID SUPPORT	NO CELL WANDERING	PER CENT POSITIVE ON SOLID SUPPORT
<i>Defibrinated serum</i>				
Spider web.	17	15	2	88
Large drop.	11		11	
Spread drop.	5	1	4	20
<i>Saline solutions</i>				
Spider web.	25 ¹	11	14	44
Large drop.	5		4 ²	
Spread drop.	5	2	3	40

¹ Exclusive of three cases in tap water in which disintegration began very early.

² In one case tissue touched bottom of chamber and some outwandering occurred.

Series III mounted respectively upon spider web and in a plain drop. In the latter (fig. 2) there are a great many loose cells which have separated from the main mass of tissue and assumed spherical shape, while the main mass itself looks to be upon the point of disintegration, though, as subsequent observation showed, this did not take place. The drawing was made about twenty-eight hours after the tissue was prepared. In figure 1 the companion preparation is represented. The mass of tissue is held against the cover-slip by the spider web and is much more flat-

²¹ Cf. M. R. and W. H. Lewis, *Anat. Rec.*, vol. 5, 1911.

tened than in the other case. A comparatively thin fringe of cells has formed around it and many cells have left the mass entirely, being scattered at various distances from the latter. These cells are at two levels. The upper layer is in contact with the lower surface of the cover-slip, where the outwandering cells are apt to assume a polygonal form, though they are sometimes influenced by the web fibers attached to the cover. In the lower layer the cells are arranged with reference to the web fibers, and they are usually drawn out into long processes which are closely applied to the latter (see also figs. 4 to 7).

The preparation shown in figure 3 is from Series IV, being one of those mounted in Locke's solution. It shows the effect of contact with the smooth cover-slip only. The drawing was made about forty-five hours after the culture was prepared. The tissue consisted of a piece of the medullary cord with some mesoderm and a small piece of the notochord attached. It has been flattened in pancake form against the cover-slip by the spreading of the drop of fluid. A small number of isolated polygonal cells have moved off upon the cover, and a thin fringe of cells surrounds the tissue as in the preceding case.

The adaptation of the cells to the web fibers is shown more clearly in figures 4 to 7, which are all taken from the same series of experiments. Figure 4 shows a cell with two hyaline processes attached to the web fibers running approximately at right angles. Figure 5 shows two bipolar and a tripolar cell, drawn when the preparation was two days old, figure 6 two cells from an eight day culture, and figure 7 pigment cells from another preparation of six days. The pigment is of two kinds, a granular black melanin and a diffuse yellow pigment (probably a lipochrome) present in two of the cells in large quantities. The yellow cells contain a little of the dark granular pigment, though much less than the other cells, and the latter contain none of the yellow.

Nerve fibers developed in only a few of the cases and in none of these was anything notable shown. All of the nerves observed were growing upon the surface of the cover slip. The web fibers do not seem to be a favorable support for them.

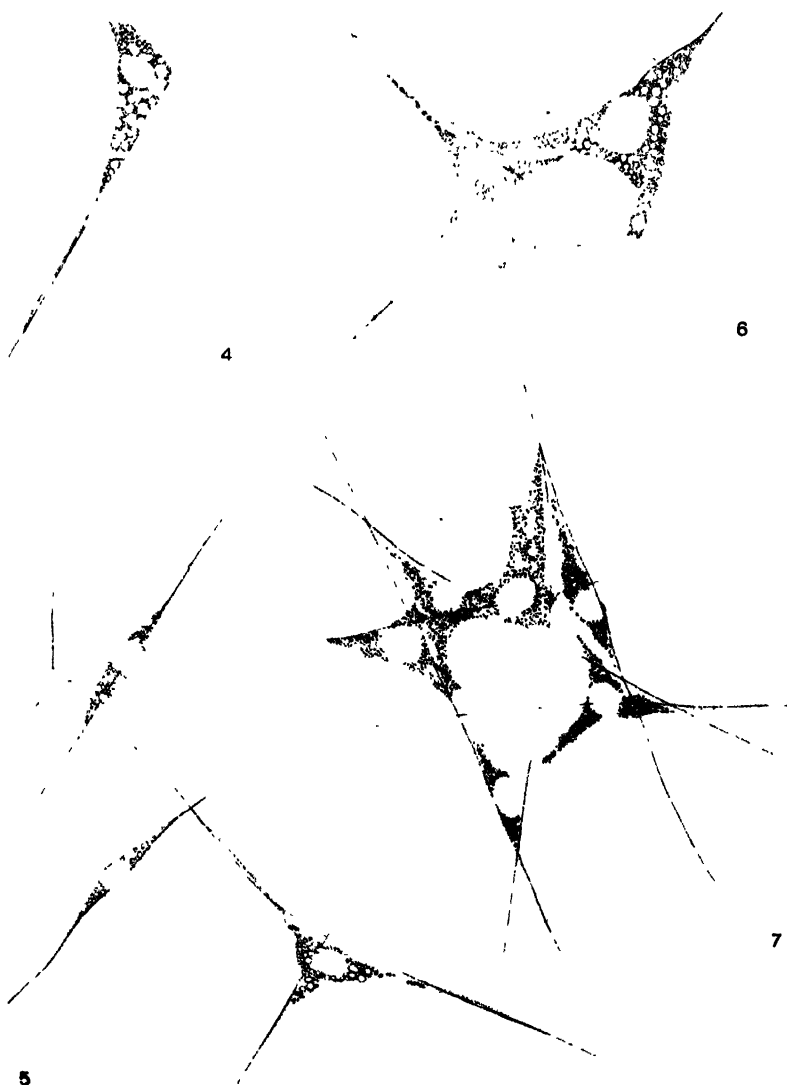


Fig. 4 Experiment S. W. 47. Cell from medullary cord with two processes attached to crossed web fibers. $\times 300$.

Fig. 5 Preparation S. W. 53. Bipolar and tripolar cells from medullary cord attached to spider web fibers two days after explantation. $\times 300$.

Fig. 6 Experiment S. W. 39. Similar preparation eight days old. $\times 300$.

Fig. 7 Experiment S. W. 49, showing the two types of pigment cells; six days old. $\times 300$.

EXPERIMENTS WITH CHICK TISSUES

One hundred and two experiments divided into ten series were made with chick tissue, comprising altogether 142 different cultures since in some cases more than one culture was made under the same cover. In three of the series the medium used was Locke's solution, and in the other seven it was defibrinated serum. Clotted plasma was used for comparison in all series. When plasma and serum were used the blood was always taken from the same hen so as to have an identical fluid medium in all cases.

Fixed support for the tissues was afforded by spider web prepared as described above, by clotted plasma and by the lower surface of the cover-slip. The latter was rendered available by mounting the tissue in small drops. In other cases contact was brought about after a time by the spreading of the drop which was originally large. This gives a firm attachment but rarely a luxuriant growth because of the extreme thinness of the fluid film.²² Tissues from embryos varying in age from two to nine days were used. The results are given briefly in table 3, which shows the behavior of cells in the different media without, however, attempting to analyze the behavior of different tissues. Plasma preparations are 100 per cent positive. The cover-slip preparations (i.e., those mounted in small drops) show also a very high percentage of positive results while the spider web cultures are less favorable, due probably as pointed out above, to the extremely small amount of fluid used. The large drop preparations are all negative except two which show some cell wandering on the surface film. Several of the small drop cultures also show slight cell movement on the surface. The film cannot, however, be a very favorable surface for movement; otherwise we should expect to find cells upon it much more fre-

²² Burrows (Trans. Am. Cong. Phys. and Surg., 1913) has shown that the amount of cell migration depends upon the thickness of the layer of medium. There is an optimum thickness above and below which less movement takes place; in the thick layer because of insufficient oxygen and lesser concentration of repelling waste products (acid), and in the very thin layer (below the optimum) because of the small amount of nutrient medium.

TABLE 3

Summary of results of experiments with chick tissues

CHARACTER OF CULTURE	NUMBER OF PREPARATIONS	CELL WANDERING ON SOLID SUPPORT	CELLS ON SURFACE FILM	NO CELL WANDERING	PER CENT POSITIVE ON SOLID SUPPORT
Plasma clot.	34	31			100
<i>Defibrinated serum</i>					
Spider web.	31	23		8	74
Large drop	14		1	13	
Small drop	5	5			100
Spread drop	6	1		5	17
<i>Locke's solution</i>					
Spider web	30	19		11	63
Large drop	7		1	6	
Small drop	12	10	2	1	83
Spread drop	3	3			100

quently. It is of interest to note that in several of the large drop preparations where the tissue was at first not in contact with the cover, it afterwards did touch the glass and then cell movement began. These have been classed with the small drop (contact) group.

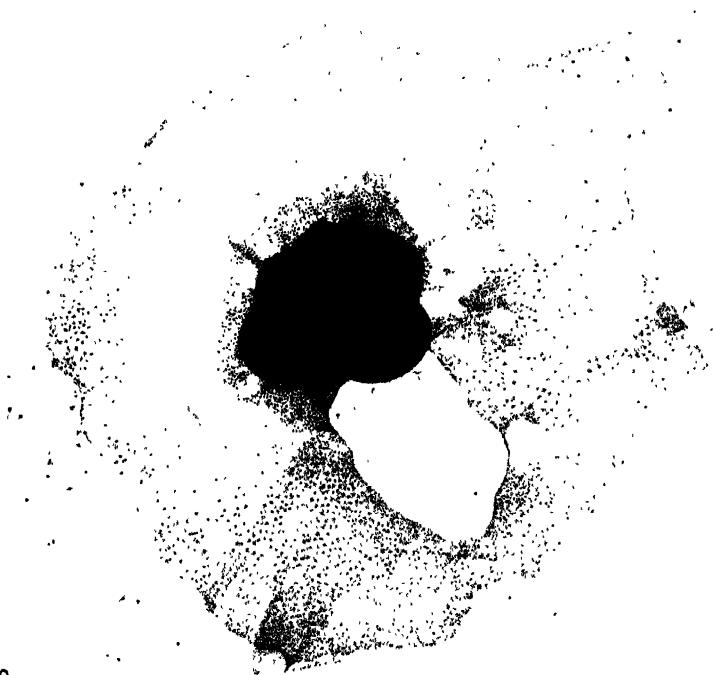
The main results of these experiments can best be presented by the description of four different cases taken from the same series and shown in figures 8 to 11. The cultures are all from pieces of the duodenum of a nine-day chick embryo, and the drawings were made from specimens preserved two days after preparation.

The first (fig. 8) has been cultivated in clotted plasma. In this there is a very characteristic ring formation with bands of tissue extending across the clear space. Isolated mesenchyme cells are present and the epithelium (endothelium?) shows its usual tendency to form membranes.

The second (fig. 9) was cultivated in a small drop of defibrinated serum and the tissue was in contact with the cover-slip from the beginning. The striking feature of this case is the formation of a wide membrane extending out from nearly the whole circumference of the original tissue. In one region conditions have prevented this movement, but at a distance from



8



9



Figs. 8 to 11²³ Duodenum of nine-day chick embryo cultivated with different kinds of solid support. Drawn from specimens preserved two days after explantation. $\times 39$.

Fig. 8 In clotted plasma.

Fig. 9 In serum in contact with cover glass.

Fig. 10 In serum on spider web.

Fig. 11 Free in large hanging drop of serum.

Fig. 12²³ Portion of figure 10 under high power. $\times 300$. Explanation of details in text.

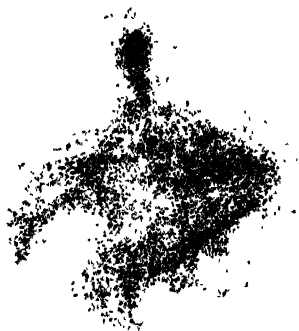
²³ These preparations, which were used to illustrate a general paper read before the American Congress of Physicians and Surgeons, have been redrawn (except fig. 31) for the present account in order to show greater exactness of detail.

the main mass the membrane from the two adjacent sides has united, leaving a large hole, and there are also several smaller spaces left uncovered. A considerable number of isolated branched mesenchyme cells are to be found outside the area covered by the membrane. All of these structures are fixed to the cover-slip. The cells forming the membrane are one layer thick.²⁴ Those near the periphery of the membrane have the greatest superficial area, and there is a general decrease in their dimensions as the tissue mass is approached, though there are irregularities in this respect. There are no mitoses present, which indicates that membrane formation is due to movement of cells and not to growth.²⁵ There is no way of ascertaining whether this particular membrane is endothelial or from the epithelium lining the intestinal tract.

The third culture (fig. 10) was supported upon spider web in the manner described above. It does not show such extensive cell wandering as some other preparations but it is very characteristic. A membrane (*m*) has been formed on the cover glass at one side of the preparation, and at another place, quite detached from the main mass, there is a small group of cells (*a*) which have moved out along the web fibers. On the opposite side of the tissue a considerable mass of cells (*b*) projects sharply outward and is firmly attached to the web. What is most interesting in this case is the banding together of spindle cells upon bundles of web fibers or upon single threads to form structures which closely simulate embryonic nerves with their sheath cells (*c*). In several places web fibers covered with such cells cross one another and here there is some accumulation of cells. Spindle shaped cells are also found singly (*e*). Some necrotic tissue (*d*) is present at places around the periphery of the main mass. The arrangement of these structures is shown in figure 12, which represents part of the preparation under higher magnification.

²⁴ Cf. M. R. and W. H. Lewis, *Anat. Rec.*, vol. 6, 1912. Also Burrows, 17th International Congress of Medicine, London, 1913; Section General Path. and Path. Anat., p. 225.

²⁵ This is in harmony with the observations of many authors on the covering of wound surfaces by epithelium.



13



14

Fig. 13 Experiment C 19. Piece of telencephalon of five-day chick embryo cultivated two days in clotted plasma, showing fringe of nerve fibers growing out into clot. $\times 39$.

Fig. 14 Experiment C 15. Piece of telencephalon of five-day chick embryo cultivated two days in defibrinated serum. $\times 39$.

In the fourth case (fig. 11), which was cultivated in a large drop where the tissue never was in contact with the cover glass, there are no evidences of cell wandering whatever. The tissue is rounded off and has an almost smooth contour.

One series of experiments was made to test the effect of the different kinds of support upon the growth of nerve fibers. Pieces of the telencephalon of a five-day embryo were used and twelve cultures were made, four of each kind, in a large drop, on spider web and in clotted plasma respectively. Those mounted in the plasma clot all showed the development of nerve fibers one day after explantation. In the more favorable cases they formed a veritable fringe around the whole periphery of the specimen. The fibers were much matted together and some were branched (fig. 13). Neither the preparations on web nor those

in the large drop showed the same condition. Two of the former showed cell movement, i.e., some wandering upon the cover and the web fibers but no nerves were present. In all of the large drop preparations (fig. 14) the contour of the tissue was relatively smooth and no cells wandered out. These experiments tend to corroborate the results obtained with frog tissues, that the web fibers are not favorable to the outgrowth of nerves, though they are too few in number to be at all conclusive in this respect.

DISCUSSION

The foregoing experiments lead to the conclusion that solid objects are an important and even necessary factor in the movement of embryonic cells, such as mesenchyme and epithelium. Leaving out the cases of movement upon the surface film, which, moreover, has certain properties of solids, there are no exceptions to the rule that movement takes place only when contact with solid material is attained. Furthermore, each of the three kinds of solid support used in the experiments influences the cell movement in its own way, as is shown by the different arrangement assumed by the cells after a certain period of cultivation (figs. 8 to 10). The reactions to solids take place whether the fluid medium is a saline solution or serum, though the latter is conducive to more active movement (and growth) than the former, owing no doubt to its nutritive qualities.²⁶

The question now arises whether these reactions are to be regarded as a manifestation of stereotropism (thigmotaxis), which is a response to mechanical stimulation (pressure), or whether the solid acts only indirectly by inducing conditions that give rise to chemical or some other form of stimulation. Burrows²⁷ has shown that the centrifugal movement of cells observed in almost all cultures, i.e., the movement from the implanted cell mass out into the culture medium, may be explained by the acidity produced in the main mass of cells through the accumulation of waste products. That a condition of acidity does

²⁶ Cf. M. R. and W. H. Lewis, *Anat. Rec.*, vol. 6, 1912.

²⁷ *Proc. Am. Cong. Phys. and Surg.*, 1913.

obtain in the center of tissue cultures after a few hours of incubation has been proved by means of indicators.²⁸ A given cell would thus be exposed on one side to a more acid medium diffusing from the tissue, and on the other to the more alkaline culture medium. This condition would, in accordance with the Quincke-Bütschli surface tension theory of amoeboid activity, bring about the formation of pseudopodia on the side of the cell turned toward the fresh medium and thus produce a centrifugal movement. Similarly the flattening of an isolated cell or the spreading of a sheet of cells on a smooth surface would be accounted for. Burrows thinks that no movement takes place as the result of mere contact with solids without the secondary stimulus resulting from the chemical change.

These considerations at least show that even under very simple conditions, where no chemical stimuli have been intentionally applied, the local activities within a culture may nevertheless give rise to such conditions as might stimulate the cells chemically on one side and thus direct cell movement. It however remains a fact that the chemical stimuli in question are powerless to call forth these movements in the absence of solid support, else the large drop cultures would not behave as they do. The acidity theory also offers no adequate explanation of the adaptation of single cells to such minute structures as the web fibers, nor of the fact that outwandering cells rapidly bridge a gap between two separate pieces of tissue in the same culture. While it must therefore be admitted that chemical stimuli may play an important part in influencing the movements of cells in simple cultures, as Burrows has pointed out, the facts show that the cells are also stimulated by solids as such and respond to them by an orienting movement.

Response to tactile stimuli is of such general occurrence in animals that there is nothing anomalous in the manifestation of the same kind of sensitiveness in cells. Though in the metazoa the responses are brought about by complex neuro-muscular mechanisms, the reactions to mechanical stimuli given by tissue cells and the protozoa are closely comparable with one another.

²⁸ Cf. Burrows, *ibid*, and Rous, *Jour. Exp. Med.*, vol. 18, 1913.

The latter have been studied by Verworn²⁹ in Rhizopods and by Pütter³⁰ in Flagellates and Ciliates. These observers have shown that different parts of the same organism respond differently to contact stimuli. In Protozoa, where the movement is sufficiently rapid to be directly observed, the location within the cell of both the sensitive region and the responding mechanism can often be made out, whereas it has as yet been found impossible to do so in the case of the slowly moving tissue cells. This is a difficulty that applies alike to the study of the reactions to all kinds of stimuli and renders uncertain for the present any attempt at exact description of the process. At the same time the case of *Chilomonas*, for instance, in which according to Pütter stimulation of one of the flagella by solids calls forth a positive response, while similar stimulation of the other a negative one, shows that we have in this kind of sensitiveness an important directive factor. By analogy in the case of tissue cells, if different parts of the same cell are unequally sensitive, it is likely that contact with an even surface would induce movement in a definite direction. Barring some such regional difference in the irritability of the cell, the kind of reaction called forth by contact with a solid object could only be a clinging to that object or a recoil from it. The former or positive reaction is that shown by the embryonic cells here studied. Without any further stimulus it is conceivable that the reaction to such contact might result in the change of shape of the cell, either its flattening upon the surface of a glass cover-slip or its elongation upon the fibers of the spider web (fig. 12). Its locomotion, however, must depend either upon some other stimulus or upon some local internal differences in the cell.

As to the propriety of calling the reaction to solids a tropism, the position here taken differs from that of Loeb who holds that it is no real tropism, in as much as lines of force do not exist.³¹ It is true that in the case of tactile stimuli the source of the

²⁹ *Allgemeine Physiologie*, 5th Edition, p. 519; *Bewegung der lebendigen Substanz*, Jena, 1892.

³⁰ *Arch. f. Anat. u. Physiol. physiol. Abt. Supplementband*, 1900.

³¹ *Dynamics of living matter*, New York, p. 156.

stimulus is not placed at a distance from the organism, for it is the relative motion of the organism stimulated and the stimulating object acting only when they come into contact, that gives rise to the stimulus, and in the case of a moving organism coming into contact with a fixed object the energy is supplied by the organism itself. However this is not sufficient ground for drawing general distinction between the response to such stimuli and the reaction to a beam of light. The difference lies only in the source of the stimulus itself, i.e., in the distance from which it comes, and in the continuousness of its effect. In the case of a negative reaction to pressure the stimulus must cease as soon as the recoil is made. In case of a positive reaction the stimulus may continue but there can be no further visible effect beyond the change in shape of the organism unless its parts are differentially sensitive. The similarity to phototropism becomes closer in the case of rheotropism or reaction to currents in the surrounding medium, which, as Verworn pointed out,³² is to be classed under the group of responses to mechanical stimuli.

A word remains to be said regarding the significance of the facts brought out in this study with reference to the interpretation of some of the phenomena of normal development. The resemblance between the arrangement of cells on the web fibers (especially as seen in figure 12) and the sheath cells of an embryonic nerve suggests that stereotropism may have something to do with the latter, though not necessarily to the exclusion of chemotropic influences. Similarly the close application of mesenchyme cells to such structures as blood vessels, muscles, and various other organs, resulting in the formation of a cellular sheath, which afterwards becomes skleratized, may be due in the first instance to a stereotropic response. In fact the conditions within the embryo at the time when the tissues are taken for the experiments are such as to suggest that this stereotropic reaction is an important factor in the behavior of many kinds of cells. There is a very active movement of mesenchyme at the time, during which cells stream from certain regions and fill in the

³² Allgemeine Physiologie.

spaces between the main organ systems of the body which are already laid down. In this process the surface of structures such as the medullary cord, notochord, alimentary canal, muscle plates and the inner surface of the epidermis would serve as a solid base upon which the cells might creep. It is found that sooner or later all of these surfaces become ensheathed or lined by connective tissue cells. In the encystment of foreign bodies within the organism a similar phenomenon is observed.

With regard to the movements of the growing nerve fiber the evidence, as pointed out above, is not quite so varied, but it is sufficient to warrant the conclusion that also this protoplasm is stereotropic. No free outgrowth of nerves in a fluid medium has ever been observed, while such solids as the fibrin clot and smooth glass surfaces serve readily to support them, as do the surfaces of the larger cell masses and the interstitial protoplasmic network inside the embryo.

Most of these points were discussed some years ago by Herbst,³³ who, however, did not claim to have reached a definite conclusion as to the exact kind of reaction involved. The experiments here described do not of course settle the question either, but since it has been shown that most embryonic cells are stereotropic, and that such arrangements as they assume in the embryo may often be induced under cultural conditions by reactions to solids, there is a presumption in favor of the view that this type of reaction is a potent factor in normal development also. Inferences as to what goes on in the embryo which are not based upon exact information regarding the physiological properties of the tissue elements are likely to prove erroneous. On the other hand, if we know the actual properties of individual cells in detail, it will be possible to form, on the basis of the observation of normal development, an accurate conception of the influences actually at work in shaping the embryonic body.

³³ Biol. Centralbl., Bd. 14, pp. 746.

THE INFLUENCE OF FOOD IN CONTROLLING SEX IN HYDATINA SENTA

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Considerable interest has been manifested concerning the sex ratio in the parthenogenetically produced male and female individuals in the rotifer *Hydatina senta*. At one time in general cultures of rotifers only females are found, at another time females and males are found in equal numbers, and at still other times very few females but from 80 to 90 per cent of males are found.

The problem has been to determine the causes that regulate the production of the two sexes in this rotifer—why at one time there is produced an excess of females and at another time an excess of males.

The results of the work of earlier investigators on this problem having been reviewed so thoroughly and frequently recently, only references to them in the bibliography will be made at this time.

Whitney made an extensive series of experiments and observations in regard to temperature and starvation and came to the conclusion that neither factor was influential in the regulation of the production of the two sexes. He, moreover, found that the so-called female sex strains of Punnett could be made to produce many males by changing the environment. However, he was unable to discover the real factor that changed the female sex strain into one that produced many males, but he was of the opinion that whatever the potent factor was that sometimes caused only females to be produced and at other times caused nearly all males to be produced, it must be an external factor. Moreover, he made observations on one strain through 289 generations, for about two years, in which no males were

produced. This was accomplished by using a continuous and uniform diet of the flagellate, *Polytoma*, reared in a solution of cooked horse manure. Throughout this time in side experiments on this strain males were produced in large numbers many times by transferring the rotifers to a new mixed food culture.

Shull carried on many experiments with temperature, starvation, and various chemicals and finally corroborated the general conclusions of Punnett and Whitney that temperature and starvation were negative factors in the regulation of the two sexes. He, however, was able to demonstrate that the unknown factor must act upon the grandmother and not upon the mother in order to cause abrupt changes in the sex ratio among the progeny. In this point he was in agreement with Maupas' work. However, Shull finally concluded that this influential factor that controlled the production of the two sexes was an *internal* one.

Mitchell in working with the rotifer, *Asplanchna*, recently has found that a continuous diet of a uniform food culture will cause only females to be produced, but if the diet is changed to a new food, males are produced in the third generation (grandchildren). He suggested that a change of food might be found to be the controlling factor in regulating the production of the two sexes in *Hydatina senta*.

In the experiments of the previous workers upon *Hydatina senta* very little attention has been given to any particular kind of food. Usually any mixed Protozoa culture upon which the rotifers would thrive was used. However, Shull and Whitney used a more or less pure culture of the colorless flagellate, *Polytoma*, but obtained varying results. This may be due to the fact that these cultures in some instances were mixed cultures to a considerable extent although not suspected to be mixed. Whitney's cultures were probably the same throughout the two-year period in which he obtained 289 generations of females. Uniform conditions produced uniform results. At that time this point was not fully appreciated.

About five years ago the author had a general culture of rotifers in a jar which was placed in a south window in the laboratory and remained there during the spring months. Some time in May during a period of a few days the jar was swarming with countless numbers of the green flagellate, *Dunaliella*, Teodor (*Chlamydomonas*, Cohn). On one day thirty-two female eggs of the rotifers were taken from the surface of the culture water and placed in watch-glasses and allowed to hatch. Each young female matured and thirty (93+per cent) of these females produced male offspring. This *Dunaliella* culture soon disappeared and no other good one ever appeared again. However, the author saved a little of the culture and spent the entire summer in the attempt to grow pure or even mixed cultures of it. No medium was found in which it would grow.

This year another attempt has been made to grow cultures of *Dunaliella* and has been quite successful although the method is not yet completely perfected. They were raised in countless numbers in direct sunlight and a solution of bouillon. An Armour's bouillon cube was dissolved and boiled in 400 cc. of tap water and equal parts of this bouillon solution and of sterilized water were used for the culture medium. The medium was inoculated with a few individuals of *Dunaliella* and the jar placed in a south window. Within 7 to 14 days the surface of the culture water was swarming with the active *Dunaliella* during the sunny part of the day. Toward night the animals became inactive and motionless, in which state they remained until the following morning and then only became thoroughly active again provided there was direct sunlight. After the culture became 2 to 3 weeks of age the *Dunaliella* would remain continually in the motionless state. At this time the old culture water was siphoned off and some new culture medium was added to the quiescent *Dunaliella*. Within 2 to 3 days, if in sunlight, practically all the *Dunaliella* would be active. The surface was removed with a pipette and placed in a test-tube and by centrifuging, all the *Dunaliella* were collected at the bottom of the test-tube. Then nearly all of the liquid in the test-tube was poured off, thus leaving the *Dunaliella* in enormous

numbers in a small amount of the culture water. Three or four drops of this material was added to about 8 cc. of old filtered culture water in which a general mixed culture of Protozoa and rotifers were living and placed in watch-glasses. These glasses were placed in sunlight at a temperature of about 25°C. and the *Dunaliella* remained active as long as they were in the sunlight.

It, however, usually happened that when these watch-glasses were left in the sunlight for any appreciable length of time, the temperature would rise to 31 to 40°C., which would be fatal to the rotifers. To obviate this difficulty, a smaller battery jar was set and fastened into a larger battery jar which had padding in the bottom of it. This allowed the top of the smaller jar to protrude above the top of the larger jar. A space was thus made between the two jars into which running water was conducted, and by regulating the flow the temperature could be kept at almost any point desired while this water-jacket jar was in direct sunlight. The rotifers in the *Dunaliella* culture were placed in the inner jar in watch-glasses and the jar was covered with a glass plate. By this method a constant temperature of about 25 to 26°C. was maintained, at which degrees the *Dunaliella* were the most active in swimming about in the culture water. The rotifers eat them only when they are active, chiefly because when they are motionless they form a scum at the surface of the culture water or become fastened to the sides of the dish and in either place they are inaccessible to the rotifers.

At the beginning of the experiments a female rotifer was taken from a jar containing a general culture of rotifers which had developed from fertilized eggs a few months previously. This female was fed upon pure cultures of the colorless flagellate, *Polytoma*, and became the progenitor of a parthenogenetic strain of rotifers which produced almost entirely female offspring through twenty-five generations. The method of causing a strain to produce only female offspring has been described in a former paper, but as the method of making the food cultures has been improved it will be briefly described again. Pure cultures of *Polytoma* were made in the following manner: 800 grams of fresh horse manure were put into 1200 cc. of water and

cooked in a steam sterilizer at 10 to 15 pounds pressure for one hour. The liquid part was then pressed out and usually equalled 1000 cc. This was put into a flask with a cotton plug and sterilized. This could be kept indefinitely and used as a stock supply provided it was sterilized each time after it was opened. One part (100 cc.) of this liquid was added to three parts (300 cc.) of sterilized water, inoculated with a few *Polytoma* and placed in a large flat dish, thus giving a large surface of the culture water exposed to the air. Within 2 to 3 days the culture water would be teeming with the *Polytoma* but after a day or two the culture became spent and only a few *Polytoma* would be found. When this culture was about three days old one-half of it was poured off and 100 cc. of the stock supply of the manure liquid and 300 cc. of sterilized water were added. Eighteen to twenty-four hours later countless numbers of the *Polytoma* could be taken off from the surface with a pipette. This process of pouring off one-half of the old culture water and adding new medium was repeated every day and in this way a vigorous food culture of *Polytoma* was continuously maintained for the rotifers at room temperature.

Female rotifers of various ages were taken from the controlled female strain, which produced nearly all females through twenty-five generations by being fed upon a continuous diet of *Polytoma*, and fed the green flagellate, *Dunaliella*, by the method as described earlier in this paper.

Tables 1 to 5 show the important results obtained in a long series of observations.

Experiments 1 to 23 have been omitted because they were made when the methods of feeding were being perfected and consequently did not show as favorable results as the later experiments. Experiments 24 to 63 include all the other experiments which were made in a successive series. A large number of individuals, considering the amount of work that each one entailed, has been observed, as recorded in tables 1 to 3. 341 mothers produced 5562 daughters, of which 3 per cent were male-producing daughters when reared under the influence of *Polytoma* diet, and of which 57% were male-producing daughters

when the diet of their mothers was changed from *Polytoma* to *Dunaliella*. In the general control experiments in which a continuous diet of *Polytoma* was maintained 177 mothers produced 2500 daughters, of which 2+ per cent were male-producing daughters.

The highest percentage of male-producing daughters which was produced by a change of the diet in the experiments in tables 1 to 3 is 83+ but as high a percentage as 87+ was produced in the experiment in table 4.

If twenty-five of the best experiments in tables 1 to 3 are selected and the percentage computed for the male-producing daughters, it is seen that a change of diet from the *Polytoma* to

TABLE 1

Showing that adult female-producing females of various ages, selected at random from a rotifer culture reared on a Polytoma diet through several generations, can be forced to produce a high percentage of male-producing daughters by being transferred from a Polytoma diet to a Dunaliella diet

EXPERIMENT	CONTROL REARED AND CONTINUED ON A POLYTOMA DIET				ADULT ♀♀ REARED ON A POLYTOMA DIET TRANSFERRED TO A DUNALIELLA DIET			
	♀ Mothers	Offspring			♀ Mothers	Offspring		
		♀♀	♂♀	♂, ♂♀		♀♀	♂♀	♂, ♂♀
24	5	30	2	6+	5	23	19	45
25	5	28	2	6+	5	27	12	30+
26	5	36	0	0	18	130	45	25+
27	2	20	0	0	2	12	8	40
28	5	17	1	5+	5	29	15	34+
29	5	30	0	0	5	20	7	25+
30	5	44	0	0	8	24	56	70
31	4	30	0	0	8	37	21	36+
32	4	24	0	0	20	100	78	43+
33	5	40	0	0	9	32	32	50
34	5	33	1	2+	12	77	49	38+
35	5	47	5	9+	22	54	211	79+
36	5	58	2	3+	20	42	172	80+
37	4	60	0	0	15	50	122	70+
38	5	65	3	4+	16	84	156	65
39	5	104	2	1+	10	64	83	56+
Total.....	74	576	18	3+	180	805	1086	57+

the Dunaliella causes the percentage of male-producing daughters to change from almost zero to 63+.

The average size of a family of daughters from each mother was 16+. The size of the families in tables 1 and 2 were intentionally made small because of the labor entailed had they been made larger. In table 3 the mothers seemed unable to bear the strain of being transferred from the Polytoma diet to the Dunaliella diet for the second time and usually soon died. It is possible that some of them were producers of small families, as Shull has shown in some of his experiments.

TABLE 2

Showing that young adult female-producing females reared on a Polytoma diet will continue to produce female-producing daughters but can be forced to produce a high percentage of male-producing daughters by being put upon a Dunaliella diet and later can be forced to produce again female-producing daughters by being put back upon the Polytoma diet

EXPERIMENT	CONTROL REARED AND CONTINUED ON A POLYTOMA DIET					YOUNG ADULT ♀♀s REARED ON A POLYTOMA DIET TRANSFERRED TO FRESH POLYTOMA DIET					SAME ADULT ♀♀s TRANSFERRED TO A DUNALIELLA DIET					SAME ADULT ♀♀s TRANSFERRED BACK TO A POLYTOMA DIET							
	Young ♀♀ mothers	Offspring				Lot	Young ♀♀ mother	Offspring				Lot	♀♀ Mothers	Offspring				Lot	Mothers	Offspring			
		♀♀	♂♂	%	♂♀			♀♀	♂♂	%	♂♀			♀♀	♂♂	%	♂♀			♀♀	♂♂	%	♂♀
40	5	54	0	1	A	5	64	0	0	A	5	26	18	40+									
41	5	58	0	0	A	5	50	0	0	A	4	18	12	40									
42	5	88	0	0	A	5	44	0	0	A	5	23	38	63+									
					B	5	44	0	0	B	5	36	32	47+									
43	5	48	0	0	A	5	45	0	0	A	5	35	13	27+									
44	5	122	32	20+	A	4	16	0	0	A	4	25	45	64+	A	4	66	4	5+				
45	5	106	2	1+	A	5	26	0	0	A	5	41	25	37+	A	5	32	0	0				
46	5	84	0	0	A	5	16	0	0	A	5	10	26	72+	A	5	24	0	0				
					B	1	2	0	0	B	1	9	7	43+	B	1	16	0	0				
47	5	99	9	8+	A	5	30	0	0	A	5	15	55	78+	A	5	14	2	12+				
48	5	100	0	0	A	5	36	0	0	A	5	34	30	46+	A	5	16	0	0				
					B	5	28	0	0	B	5	35	35	50	B	5	26	0	0				
49	5	106	0	0	A	5	18	0	0	A	5	34	14	29+	A	5	42	0	0				
					B	1	12	0	0	B	1	10	6	37+	B	1	17	1	5+				
50	5	72	0	0	A	5	26	0	0	A	5	28	36	56+	A	5	26	0	0				
					B	5	28	0	0	B	5	42	26	38+	B	5	24	0	0				
Total	55	937	43	4+		71	485	0	0		70	420	418	49+		46	303	7	2+				

The temperature was uniform for all the individuals of each experiment. When the individuals were put upon a *Dunaliella* diet they were kept at 25 to 26°C. and the control individuals were also kept at this same temperature. During the other time both before and after the change in diet all individuals were kept at room temperature of 18 to 20°C.

The results of Maupas are very easily explained in the light that is given by these experiments. Maupas probably used a

TABLE 3

Showing that young adult female-producing females reared on a Polytoma diet can be forced to produce a high percentage of male-producing daughters among the first children of each family by being put upon a Dunaliella diet and later these same adult females can be forced to produce a high percentage of female-producing daughters by being changed back to a Polytoma diet and still later they can be forced to produce again male-producing daughters by being put back upon a Dunaliella diet

EXPERIMENT	CONTROL REARED AND CONTINUED ON A POLYTOMA DIET				YOUNG ADULT ♀'S REARED ON A POLYTOMA DIET TRANSFERRED TO A DUNALIELLA DIET				SAME ADULT ♀'S TRANSFERRED BACK TO A POLYTOMA DIET				SAME ADULT ♀'S TRANSFERRED AGAIN TO A DUNALIELLA DIET			
	Young adult ♀'s	Offspring	Lot		Young adult ♀'s	Offspring	Lot		Adult ♀'s	Offspring	Lot		Adult ♀'s	Offspring	Lot	
	♀	♂	♀	%	♀	♂	♀	%	♀	♂	♀	%	♀	♂	♀	%
53	5 98	22	A		5 9 39	81+	A		5 28	2 6+						
			B		5 9 47	83+	B		5 17	7 29+						
54	5 106	43+	A		5 21 17	44+	A		5 15	7 31+						
			B		5 11 19	63+	B		5 41	9 18						
55	5 130	00	A		5 17 17	50	A		5 53	7 11+	A		5 75	16 17+		
			B		5 10 18	64+	B		5 51	7 12+	B		5 85	13 13+		
56	5 75	00	A		5 26 22	45+	A		5 36	4 10	A		5 19	6 23+		
			B		5 29 15	34+	B		5 34	2 5+						
57	5 78	11+	A		5 19 37	66+	A		5 14	4 22+	A		1 2	2 50		
			B		5 27 31	53+	A		5 28	4 12+	B		4 25	17 40+		
58	5 142	21+	A		5 15 39	72+	A		5 35	3 7+						
			B		5 36 50	57+	B		5 61	3 4+						
59	5 100	43+	A		5 36 48	57+	A		5 60	0 0						
			B		5 56 56	50	B		5 64	0 0						
60	5 104	00	A		5 25 41	62+	A		5 47	1 2+						
			B		5 22 16	42+	B		5 64	0 0						
61	5 80	00	A		5 30 30	50	A		5 60	0 0						
			B		5 16 12	42+	B		5 42	0 0						
Total	45 913	131+			90 414	554			90 750	60 7+			20 206	54 20+		

mixed food culture of Protozoa. When this food culture was placed at a low temperature of 12 to 14°C. only certain species of Protozoa were active and consequently could be used as food by the rotifers. However, when the temperature of this same food culture was raised to 26+°C., other species of Protozoa which had been in a quiescent stage while the culture was at 12 to 14°C., now became active and were used as food by the rotifers instead of the Protozoa that were used at the low temperature. Thus by changing the temperature the diet was so markedly changed that it constituted the necessary stimulus upon the mothers for the production of male-producing daughters.

TABLE 4

Showing that the influence of the diet acts upon the mother and not upon the male-producing daughter that causes the daughter to produce males. In other words, the influence of the change of diet acts solely upon the grandmother and causes her to beget male grandchildren. Young ♀♀ reared on a Polytoma diet were transferred to a Dunaliella diet for 27 to 36 hours. At the end of this period they were transferred to filtered culture water and allowed to produce eggs. These eggs were transferred to Polytoma culture in which they hatched and the young females grew to maturity and reproduced while being fed exclusively upon Polytoma.

♀♀ MOTHERS REARED ON A POLYTOMA DIET	OFFSPRING FROM EGGS WHICH DEVELOPED ON A DUNALIELLA DIET BUT FED POLYTOMA AFTER HATCHING		
	♀♀	♂♂	% ♂♂
Several.....	8	16	66+
Several	9	63	87+

TABLE 5

As a control for table 4. Young ♀♀ reared on Polytoma diet were transferred to a Dunaliella diet for 26 hours. At the end of this period they were transferred to filtered culture water and allowed to produce eggs. These eggs were transferred to fresh Dunaliella culture in which they hatched and the young females grew to maturity and reproduced.

♀♀ MOTHERS REARED ON A POLYTOMA DIET	OFFSPRING FROM EGGS WHICH DEVELOPED ON A DUNALIELLA DIET AND FED DUNALIELLA AFTER HATCHING		
	♀♀	♂♂	% ♂♂
.....	25	25	41+
.....	30	10	25

The author has several records of epidemics of males occurring in his experiments during the last eight years at periods when accidental rises of temperatures from $20^{\circ}+$ to 26° or 27°C. took place and while the food used was a mixed culture of green and colorless Protozoa. These epidemics of males can be explained in the same manner as the results of Maupas. At room temperature certain species of green Protozoa were more or less quiescent but when the temperature rose suddenly to $26^{\circ}+\text{C.}$ all the individuals of these somewhat quiescent green Protozoa became very active and furnished a new diet for the rotifers. The stimulation by this new diet caused the mothers to produce male-producing daughters. After a few hours the temperature sank back to the normal temperature of $20^{\circ}+\text{C.}$ and the remaining green Protozoa again became quiescent and the rotifers were forced to eat the other Protozoa that were normally active at this temperature and which was their regular diet.

It has been previously observed that rotifers in a newly made general culture of manure medium produce a much higher percentage of male individuals than rotifers in an old culture of manure medium. It is generally known that in a newly made hay infusion—and the same is true in a newly made manure infusion—the protozoan fauna fluctuates greatly. At first individuals of certain species may be very abundant and later individuals of other species became very numerous, while the individuals of the earlier-appearing species in the culture become relatively few. Thus there is a never-ending change in the protozoan fauna in a new culture of water and manure. Certain species flourish and are very abundant for a short period and then they disappear and new forms replace them. When rotifers are in such culture water with its varying protozoan fauna they are, of course, subjected to many changes of diet. Some of these changes of diet probably act as a stimulus upon the female rotifers so as to cause them to produce male-producing daughters which produce males in the following generation.

The sporadic production of males in the numerous experiments of various workers who have used mixed protozoan cultures as

food for the rotifers can thus be simply explained. Under certain conditions some of the Protozoa are active and others are more or less quiescent, although they may be reproducing in this quiescent stage. When the conditions are changed, possible in other ways than temperature, the quiescent Protozoa become very active, thus constituting a new diet for the rotifers which eventually causes males to be produced.

It would be interesting to know the effect of a continuous feeding of a new diet upon the rotifers, but unfortunately it has been impossible to use the green *Dunaliella* continuously as a diet. They are the most active in sunlight but during the night they become more or less quiescent and consequently can not be used as a food by the rotifers in any appreciable numbers.

It is very probable that other forms of Protozoa as well as *Dunaliella* have the same stimulating effect upon female-producing females of *Hydatina senta* in causing them to produce male grandchildren because in mixed colorless protozoan cultures epidemics of males often occur. It may be possible to find and cultivate a colorless flagellate which will be even more effective than the green one, *Dunaliella*, which has already been used and caused the female-producing females to yield as high as 87 + per cent of male-producing daughters.

The sex strains of Punnett may be more or less due to the diet used. Punnett states that they were fed upon *Euglena* most of the time but does not state whether the *Euglena* were in pure cultures. In some of the food cultures made this year an undetermined species of *Euglena* was cultivated in the same kind of bouillon solution as was *Dunaliella*. This was an excellent food for the rotifers but it did not stimulate them to produce males, as the *Dunaliella* always did when the rotifers were suddenly transferred to it from a *Polytoma* diet. In a previous paper it has been shown that a constant and uniform food supply caused one family to produce only female offspring through 289 generations, although males were produced in side experiments when the food was changed. Punnett unwittingly may have used a pure *Euglena* culture as food for some of his

strains and mixed cultures for other strains, or he may have used a mixed food culture whose protozoan fauna was stable or varied according to the light conditions or possibly other conditions in the laboratory.

In order to cause rotifer mothers to produce male-producing daughters by changing the diet from *Polytoma* to *Dunaliella* the latter diet must be very abundant so that the mothers may consume enormous quantities of it. A change to a meagre diet of *Dunaliella* causes no male-producing daughters to be produced. This fact that the *Dunaliella* diet must be very copious in order that male-producing daughters may be produced, rather indicates that a sudden change in metabolism in which the processes are carried on at their maximum rate, may be a necessary accompanying stimulus, coupled with the new diet stimulus that causes male-producing daughters to be produced.

SUMMARY

1. In the parthenogenetic reproduction of *Hydatina senta* the influence of the diet acting upon the grandmother determines the sex of the grandchildren.

2. A continuous diet of the colorless flagellate, *Polytoma*, causes female grandchildren to be produced.

3. A sudden change of the diet from *Polytoma* to an abundant supply of the active green *Dunaliella* causes male grandchildren to be produced.

4. The regulation of the sex ratio in the parthenogenetic reproduction of *Hydatina senta* therefore can be controlled by food conditions.

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